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(54) Title: MODIFIED PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 AND METHODS BASED THEREON

**(57) Abstract:** The present invention is based upon the discovery that modified plasminogen activator inhibitor type-I (PAI-1) in which two or more amino acid residues that do not contain a sulphydryl group have been replaced with amino acid residues that contain a sulphydryl group and, therefore, forms intramolecular disulfide bonds, have increased in vivo half-life. Also disclosed are the modified PAI-1 proteins, derivatives and analogs thereof, specific antibodies, nucleic acid molecules and host cells. Methods for producing modified PAI-1, derivatives and analogs are also provided. The invention further relates to Therapeutics, pharmaceutical compositions and method of using the composition for treatment. The invention may be used to inhibit angiogenesis in a subject, thereby treating diseases or conditions associated with undesired angiogenesis and cell proliferation. Such conditions include psoriasis, chronic inflammation, tumor invasion and metastasis and conditions in which angiogenesis is pathogenic. The modified PAI-1 molecules of the present invention are useful for the treatment, prophylaxis, management and amelioration of cardiovascular diseases such as, but not limited to those that are related to hyperfibrinolysis, hemophilia, and vessel leakage syndrome.

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**MODIFIED PLASMINOGEN ACTIVATOR INHIBITOR  
TYPE-1 AND METHODS BASED THEREON**

The invention was made with Government support under Department of Defense DAMD 17-01-1-0553. The government has certain rights in the invention.

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**1. FIELD OF THE INVENTION**

The present invention relates to modified plasminogen activator inhibitor type-1 (PAI-1), and derivatives and analogs thereof, in which two or more amino acid residues that do not contain a sulphydryl group have been replaced with amino acid residues that contain a sulphydryl group so that intramolecular disulfide bonds can be formed. These modified PAI-1 molecules, and derivatives and analogs thereof, have increased *in vivo* half-life of the active form of PAI-1. Also disclosed are peptides of such modified proteins, antibodies that specifically bind the PAI-1 molecules, derivatives and analogs thereof, nucleic acid molecules, particularly DNA, encoding the modified proteins, and host cells containing such nucleic acids. Methods for producing modified PAI-1, and derivatives and analogs thereof, are also provided. The invention further relates to pharmaceutical compositions comprising modified PAI-1 molecules of the invention and methods of using these pharmaceutical compositions for treatment. The invention may be used to inhibit angiogenesis in a subject, thereby treating diseases or conditions associated with undesired angiogenesis, and also provides methods to inhibit cell proliferation. Such conditions include psoriasis, chronic inflammation, rheumatoid arthritis, inflammatory bowel disease, asthmas, and other inflammatory conditions, tumor invasion, primary and metastatic neoplastic diseases (e.g., cancer), and other conditions in which cell proliferation and/or angiogenesis is pathogenic. The invention may also be used to treat cardio-vascular diseases such as, but not limited to those that are related to hyperfibrinolysis, hemophilia, and vessel leakage syndrome.

**2. BACKGROUND OF THE INVENTION**

Metastasis is the cause of most cancer-related deaths. The proteolytic degradation of the extracellular matrix is recognized as a mechanism that plays an important role in the metastatic process. Proteolytic enzymes are required to mediate tumor cell invasion into adjacent tissues and initiate the metastatic process. Urokinase plasminogen activator (uPA) is commonly overexpressed by many different human cancers. Conese et al., 1995, Clinical Hematology 8(2):365-389.

cytokines (Saksela, *supra*; Dano et al., *supra*). Following secretion, PA activity can be regulated both positively and negatively by a number of specific protein-protein interactions. Activity can be enhanced or concentrated by interactions with fibrin (Hoylaerts M et al., *J Biol Chem*, 1982, 257:2912-2919), the uPA receptor (uPAR) (Ellis V et al., 5 *Semin Thromb Hemost*, 1991, 17:194-200), the tPA receptor (tPAR) (Hajjar K A et al., 1990, *J Biol Chem*, 265:2908-2916), or the plasminogen receptor (Plow E F et al., 1991, *Thromb Haemost* 66:32-36).

PA activity can be downregulated by specific PA inhibitors (PAIs) (Lawrence, D. A et al., In: *Molecular Biology of Thrombosis and Hemostasis*, Roberts, H. R. et al., (Eds.), 10 Marcel Dekker Inc., New York, chapter 25, pp. 517-543 (1995)). In addition, PA activity is dependent on its location or microenvironment and may be different in solution (e.g., circulating blood) as compared to a solid-phase (e.g., on a cell surface or in the extracellular matrix (ECM)). The overall activity of the PA system is determined by the interactions among these various elements and the balance between the opposing activities of enzymes 15 and inhibitors.

Urokinase plasminogen activator's major function is in tissue-related proteolysis, and is important in the processes that entail the dissolution of the extracellular matrix and invasion of the basement membranes. It is produced by cells and is present in extracellular fluid in the form of an inactive, single chain proenzyme (pro-uPA). Conversion of pro-uPA 20 to active two-chain uPA by catalytic amounts of plasmin is a crucial regulatory step in plasminogen activation. This conversion provides active uPA and enables an autocatalytic acceleration of uPA formation, (Mayer, M., 1990, *Biochemical and Biological Aspects of the Plasminogen Activation System*, *Clin. Biochem*, 23:197-211) uPA is over expressed on the surface of cancer cells when compared with their normal noncancerous counterparts or 25 normal physiological levels of this enzyme.

## 2.2. PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 (PAI-1)

PAI-1 is considered one of the principal regulators of the plasminogen activator system. It is a single chain glycoprotein with a molecular weight of 50 kDa (Van Mourik J A et al., *J Biol Chem* (1984) 259:14914-14921) and is the most efficient inhibitor known of 30 the single- and two-chain forms of tPA and of uPA (Lawrence D et al., *Eur J Biochem* (1989) 186:523-533). PAI-1 also inhibits plasmin and trypsin (Hekman C M et al.,

cleavage of the secretion signal sequence, provide proteins with overlapping amino-terminal sequences of Ser-Ala-Val-His-His and Val-His-His-Pro-Pro (Lawrence et al., 1989, supra). This latter sequence is generally referred to as mature PAI-1.

PAI-1 is a glycoprotein with three potential N-linked glycosylation sites containing 5 between 15 and 20% carbohydrate (Van Mourik J A et al., supra). PAI-1 produced in E. coli, although nonglycosylated, is functionally very similar to native PAI-1. Recombinant PAI-1 can be isolated from E. coli in an inherently active form (see Section 2.2.2.), which contrasts with PAI-1 purified from mammalian cell culture (Lawrence et al., 1989, supra; Hekman et al, 1988, supra).

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## 2.2.2. ACTIVE AND LATENT CONFORMATION

PAI-1 exists in an active form as it is produced by cells and secreted into the culture medium and an inactive or latent form that accumulates in the culture medium over time (Hekman C M et al., J Biol Chem (1985) 260:11581-11587; Levin E G et al., Blood (1987) 70:1090-1098). The active form spontaneously converts to the latent form with a half-life of 15 about 1 h at 37 °C. (Lawrence et al., supra; Hekman et al., supra; Levin E G et al., 1987, supra). PAI-1 is a specific and fast acting inhibitor of both the tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Like other serpins, PAI-1 has a reactive center located on a highly strained exposed loop near the C-terminus of the molecule. The "reactive center" of PAI-1 contains the "bait" peptide bond between residues R(346) and 20 M(347) (M-methionine, R-arginine), *i.e.*, the P1-P1' residues. This bond mimics the R(560)-V(561) (V-valine) bond of plasminogen, which is the bond cleaved by the plasminogen activators during the activation of plasminogen to plasmin. On the basis of the mechanism of the action of other serpins, it has been postulated that PAI-1 binds specifically to the plasminogen activators like substrates (Seetharam R., et al., 1992, 25 Biochemistry, 31:9877-9882).

The structure of active PAI-1 differs significantly from its inactive form. The latent form of PAI-1 has a greater number of residues from the strained reactive center loop inserted between the A3 strand and the A5 strand in the deducted structure of PAI-1, leading to a collapse of the strained loop and loss of inhibitory activity. Latent PAI-1 is 30 inactive because part of its reactive center loop is inaccessible or does not have conformation to bind to its cognate proteases. The residues expected to interact with Ala 357 to Glu 362 all reside in the extended loop on the surface of the molecule. The helix D

reverses to its latent form again, as quickly as described above. In general, although theoretically useful in the treatment of cancer and other conditions, uPA inhibitors are toxic or labile. PAI-1 is not toxic but the active form has a short half-life. Hence, there is a need to provide a modified PAI-1 in which the active form has a longer half-life.

5

### 3. SUMMARY OF THE INVENTION

The present invention is based upon the observation of the present inventors that a modified PAI-1 protein in which two or more amino acid residues that do not contain a sulphydryl group have been replaced with amino acid residues that contain sulphydryl groups such as, but not limited to, cysteine residues, that form one or more intramolecule disulfide bonds, has a much longer *in vivo* half-life of the active form of PAI-1 than a wild-type PAI-1 protein, in which there is no such disulfide bond or other introduced stabilizing modification (Chorostowska-Wynimko et al., 2003, Molecular Cancer Therapeutics 2:19-28). These modified PAI-1 proteins are generated by amino acid substitutions of certain amino acid residues with cysteine residues to produce disulfide bridges linking the A3 strand and the A5 strand of the  $\beta$ -sheet of the PAI-1 protein (see Figure 6). The mutations introduced into the PAI-1 protein do not significantly reduce the PAI-1 enzymatic activity (or does not reduce the enzymatic activity to an extent that is not compensated by the increase in *in vivo* half-life) but can increase the half-life of the active form of the modified PAI-1 when compared to wild-type PAI-1.

20        Inhibitors of urokinase plasminogen activator (uPA) strongly suppress angiogenesis and consequently limit cancer growth. The majority of known small-molecular inhibitors of uPA are toxic and nonspecific, while plasminogen activator inhibitor type 1 (PAI-1) is non-toxic and highly specific for uPA. Unfortunately, PAI-1 converts to a latent, inactive form with a half-life ( $t_{1/2}$ ) of approximately 2 hrs. The present invention is aimed at  
25 providing modified PAI-1 proteins (and functionally active fragments, derivatives and analogs thereof) ("collectively, "modified PAI-1 molecules") with increased *in vivo* half-life of the active form of modified PAI-1 (as determined, for example, in Section 5.6), and preferably, with increased binding to uPA. By introducing amino acid residues that contain a sulphydryl group into the PAI-1 polypeptide such that intramolecular disulfide bonds are  
30 formed, the  $t_{1/2}$  of PAI-1 can be extended to therapeutically desired values.

Active PAI-1 has a reactive loop containing a P1-P1' site (Arginine 346 and Methionine 347 which form the "bait" i.e., mimics plasminogen) which can extend up to 18

stabilize or restrain the A3 and A5 strands of the  $\beta$ -sheet preventing the insertion of the active loop, i.e., the A4 strand, between the A3 and A5 strands of the  $\beta$ -sheet. Most preferably, the modified PAI-1 molecules have increased in vivo half-life of the active form of modified PAI-1, for example but not limited to, by virtue of disulfide bridges formed between the A3 strand and the A5 strand of the  $\beta$ -sheet. The disulfide bridges may be formed, most preferably, by introducing two, four, or six cysteine residues located near or within the  $\beta$ -sheet. The methods of making the foregoing by chemical synthesis or recombinant DNA technology are within the scope of the invention and are routine in the art.

10        In another preferred embodiment, the present invention provides modified PAI-1 proteins having at least two or more amino acid substitutions in the sequence from amino acid positions 10-40, 70-120, 150-220, 300-342, 343-350, or 351-402 of SEQ ID NO:2, more preferably in positions 31, 97, 192, 197, 347, 355 of SEQ ID NO:2 (or analogous residues in another PAI-1 molecule as determined, for example, by sequence alignment). In 15 another embodiment, the present invention provides modified PAI-1 proteins having at least two or more amino acid substitutions in the sequence from amino acid positions 1-40, 41-120, 121-220, 221-290, 300-342, 343-350, or 351-402 of SEQ ID NO:2. In more preferred embodiments, one or more pairs of amino acid residues selected from the pairs Valine 31 and Alanine 97, or Leucine 192 and Valine 347, or Glutamine 197 and Glycine 355, are 20 both replaced with cysteine residues. In a more preferred embodiment, Valine 31, Alanine 97, Leucine 192 and Valine 347 are replaced with cysteine residues. In more preferred embodiments, one or more disulfide bridges are formed between amino acid positions 31 and 97, 192 and 347, or 197 and 355. In preferred embodiments, the modifications result in holding the A3 strand and A5 strand of the  $\beta$ -sheet close together. In specific embodiments, 25 the distance between the A3 strand and A5 strand of the  $\beta$ -sheet is less than 0.5 Å, more than 0.5 Å and not more than 2 Å, more than 2 Å and not more than 4 Å, more than 4 Å and not more than 10 Å, more than 10 Å and not more than 20 Å, or more than 20 Å and not more than 40 Å. In preferred embodiments, one or more disulfide bridges are formed at or near amino acid positions 10-40, 70-120, 150-220, 300-350, or 351-400. Most preferably, 30 one or more disulfide bridges are formed at or near residues 180-370.

The present invention relates to modified PAI-1 proteins and functionally active fragments, derivatives and analogs thereof ("modified PAI-1 molecules"), preferably having one or more substitutions of amino acid residues (as described hereinbelow) that

decrease invasion and metastasis. In a preferred embodiment, modified PAI-1 molecules are used as anti-angiogenic agents to reduce tumor growth, and to inhibit primary and metastatic neoplastic diseases. In a preferred embodiment, modified PAI-1 molecules are used to block uPA proteolysis. The modified PAI-1 molecules of the invention may also be  
5 used to treat cardio-vascular diseases such as, but not limited to those that are related to hyperfibrinolysis, hemophilia, and vessel leakage syndrome.

#### 4. DESCRIPTION OF THE FIGURES

Figures 1A-D. The nucleotide sequence (SEQ ID NO:1) encoding human PAI-1 plus 5' and 3' untranslated regions from a particular clone. Also shown is the amino acid  
10 sequence of full length human PAI-1 including the signal peptide (SEQ ID NO:2).

Figures 2A-2B. (A) The amino acid sequence of the PAI-1 protein (SEQ ID NO:2) including the signal peptide. (B) The amino acid sequence of the mature PAI-1 protein (SEQ ID NO:3).

Figure 3. Percentage activity of wild-type PAI-1 vs. time elapsed as determined by  
15 amidolytic assay.

Figure 4. Percentage activity of a modified PAI-1 protein with cysteine residue substitutions at amino acid positions 192 and 347 ( $\beta$ -sheet b) vs. time elapsed as determined by amidolytic assay.

Figure 5. Percentage activity of a modified PAI-1 protein with cysteine residue  
20 substitutions at amino acid positions 197 and 355 ( $\beta$ -sheet t) vs. time elapsed as determined by amidolytic assay.

Figures 6A & 6B. (A) Ribbon model of latent form of PAI-1. (B) Ribbon model of active form of a modified PAI-1. On the left side amino acids that were mutated are shown as CPK model (balls), on the right, disulfide bridges are shown in CPK model. From the  
25 bottom: Cys 31 — Cys 97 (helix D) bridge reduces flexibility of PAI-1 molecule by holding together N terminal to the nearest helix; Cys 192 - Cys 347 ( $\beta$  sheet b) bridge tightly holds A3-A5 strands, most upper one: Cys 197 - Cys 355 ( $\beta$  sheet t) ends stiffening the reactive loop and preventing its backtracking into the  $\beta$  sheet. Models of active PAI-1 and latent form were acquired from X-ray analysis, PAI-1-VHLH was built using CHAIN program.  
30 Please note that all amino acids are numbered as in notation commonly accepted for

therapeutic and prophylactic methods using modified PAI-1 molecules of the invention. The present inventors have designed and made modified PAI-1 molecules that have mutations (preferably amino acid substitutions) in or close to the  $\beta$ -sheet of PAI-1 which increase the bioactivity of PAI-1, in particular, to increase the half-life of the PAI-1 active form in circulation. The invention provides modified PAI-1 molecules in which two or more amino acid residues that do not contain a sulphydryl group have been replaced with amino acid residues that contain sulphydryl groups such as, but not limited to, cysteine residues, so that these sulphydryl containing residues form one or more intrachain disulfide bonds, which modified PAI-1 molecules have a much longer half-life than a wild type PAI-1 protein.

In preferred embodiments, the amino acid residues to be substituted with sulphydryl containing groups are amino acids having aliphatic side chains such as, but not limited to, glycine, alanine, valine, leucine, isoleucine. In other preferred embodiments, the amino acid residues to be substituted with sulphydryl containing groups are amino acids having amide side chains such as, but not limited to, asparagine or glutamine. The disulfide bonds introduced into PAI-1 preferably hold the A3 strand and A5 strand of the  $\beta$ -sheet together so as to prevent the insertion of A4 strand between the A3 strand and A5 strand of the  $\beta$ -sheet. As discussed above, the helix D loop comprises amino acids at positions 92-107. The A3 strand comprises amino acids at positions 341-353. The A4 strand comprises amino acids at positions 353-374. The A5 strand comprises amino acid at positions 180-197. In particular preferred embodiment, the modified PAI-1 molecule has two or more amino acid substitutions with a sulphydryl group in pairs at amino acids 31 and 97, 192 and 347, or 197 and 355 of the human PAI-1 (as depicted in Figure 2A (SEQ ID NO:2), or analogous residues in other PAI-1 proteins as determined by sequence alignment). And most preferably, the modified PAI-1 molecule is modified to increase *in vivo* half-life of the active form of the modified PAI-1 molecule, for example but not limited to, forming disulfide bridges between the A3 strand and the A5 strand of the  $\beta$ -sheet. The disulfide bridges may be formed most preferably by introducing two, four, or six cysteines located near or within the  $\beta$ -sheet.

The methods of making the foregoing by chemical synthesis or recombinant DNA technology are within the scope of the invention and the skill in the art.

In another preferred embodiment, the present invention provides a modified PAI-1 molecule having at least one amino acid substitution at one of amino acid positions 10-40,

particular embodiment, the active form of PAI-1 molecule is locked in the active form over an extended period of time for over 2 weeks, 4 weeks, or 2 months.

In other embodiments, the modified PAI-1 molecules retain 100%, greater than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10% of activity compared to a wild-type PAI-1 protein.

The present invention also relates to modified PAI-1 molecules having one or more deletions or insertions of amino acid residues (as described hereinbelow) in which the active form of the modified PAI-1 molecules have increased *in vivo* half-life. In preferred embodiment, the modified PAI-1 molecules are more active than the wild-type PAI-1, for example, but not limited to, binding to uPA or tPA, and active form of the modified PAI-1 molecules have prolonged half-lives in circulation. In certain embodiments, the modified PAI-1 molecules are 1.5, 2, 3, 10, or 20 times more active than the wild-type PAI-1.

Modified molecules, fusion proteins, and nucleic acid molecules encoding such molecules, and production of the foregoing molecules, e.g., by recombinant DNA methods, are also provided.

In particular aspects, the invention provides amino acid sequences of modified PAI-1 molecules, which are otherwise functionally active. "Functionally active" modified PAI-1 protein as used herein refers to that material displaying one or more known functional activities associated with the wild-type protein, e.g., binding to uPA or tPA; or inactivation or inhibition of uPA or tPA activity, internalization of uPA/PAR complex, antigenicity (binding to an anti-PAI-1 antibody), immunogenicity, or eliciting production of anti-PAI-1 antibodies, etc.

In specific embodiments, the invention provides fragments of modified PAI-1 consisting of at least 6 amino acids, 10 amino acids, 50 amino acids, 75 amino acids, 100 amino acids, 150 amino acids, 200 amino acids, 250 amino acids, 300 amino acids, or 349 amino acids. In various embodiments, the modified PAI-1 comprises or consists essentially of a mutated helix D,  $\beta$ -sheet b,  $\beta$ -sheet t or combinations thereof.

The present invention further provides nucleic acid molecules comprising nucleotide sequences that encode modified PAI-1 proteins, and methods of using the nucleic acid molecules, for example for recombinant production of the modified PAI-1 proteins of the

methionine residue. The number of amino acid residues that may be substituted in a modified PAI-1 being 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 and less than 20, more than 20, preferably, an even number of amino acid residues are substituted.

According to the invention, the modified PAI-1 molecules comprise substitutions, 5 deletions or insertions, of two, four, six, or more amino acid residues with respect to the wild-type protein.

In one embodiment, the modified PAI-1 molecule has one or more substitutions of amino acid residues relative to the wild type PAI-1 protein, preferably, two or more amino acid substitutions in the amino acid residues selected from among residues at positions 10-10 40, 70-120, 150-220, or 300-400 of SEQ ID NO:2. More preferably in positions 31, 97, 192, 197, 347, 355 of SEQ ID NO:2 (or analogous residues in another PAI-1 molecule as determined, for example, by sequence alignment). In a preferred embodiment, the modified PAI-1 molecule comprises at least one amino acid substitution in the sequence from amino acid positions 29-32, 92-107, 180-197, 341-353, 353-374, 246-249, or 381-391.

15 In more preferred embodiments, one or more pairs of amino acid residues selected from the pairs Valine 31 and Alanine 97, or Leucine 192 and Valine 347, or Glutamine 197 and Glycine 355 are each replaced with cysteine residues. In more preferred embodiments, one or more disulfide bridges are formed at amino acid positions 31 and 97, 192 and 347, or 197 and 355.

20 In other embodiments, the modified PAI-1 molecule comprises other amino acid changes as well as long as the PAI-1 activity and/or half life of the active form of the modified PAI-1 molecule are increased.

25 In one particular non-limiting set of embodiments, other covalent bonds may be formed that hold the A3 strand and the A5 strand of the  $\beta$ -sheet closer together, thus preventing the insertion of the A4 strand between the A3 strand and the A5 strand of the  $\beta$ -sheet. Methods of chemical cross-linking include, for example, using an amine-sulphydryl cross-linker such as N-( $\alpha$ -maleimidooacetoxy)-succinimide ester ("AMAS") or N-( $\kappa$ -maleimidoundecanoyloxy)-sulfosuccinimide ester ("KMUS") (Pierce Chemical Co.). Such methods would generally involve reductive methylation of one protein to block N-termini, 30 cross-linking of blocked peptide at pH 6.5-7.5 using suflo-KMUS or AMAS, and reacting the succinimide group of the modified protein with the other protein at pH 8-9. Other

### 5.3. THERAPEUTIC AND PROPHYLACTIC METHODS FOR CANCER/DISORDERS ASSOCIATED WITH INCREASE ANGIOGENESIS

The invention provides for methods of treatment, prophylaxis, management or amelioration of one or more symptoms associated with the disease, disorder using modified PAI-1 molecules of the invention. These diseases and disorders include, but are not limited to, diseases or disorders related to angiogenesis or other functions mediated or influenced by PAI-1, uPA, or tPA, including but not limited to cell proliferation, cell attachment, cell migration, granulation tissue development, primary and metastatic neoplastic diseases (e.g. cancer), and/or inflammation, cardiovascular disease, stroke, ischemia, or atherosclerosis.

10 The present invention encompasses methods for treating or preventing diseases and disorders wherein the treatment or prevention would be improved by administration of the modified PAI-1 molecules of the present invention.

In one embodiment, "treatment" or "treating" refers to an amelioration of disease or disorder, or at least one discernible symptom thereof. In another embodiment, "treatment" or "treating" refers to an amelioration of at least one measurable physical parameter associated with disease or disorder not necessarily discernible by the subject. In yet another embodiment, "treatment" or "treating" refers to inhibiting the progression of a disease or disorder either physically, e.g., stabilization of a discernible symptom, physiologically, e.g., stabilization of a physical parameter, or both. In yet another embodiment, "treatment" or "treating" refers to delaying the onset of a disease or disorder.

In certain embodiments, the methods and compositions of the present invention are useful as a preventative measure against disease or disorder. As used herein, "prevention" or "preventing" refers to a reduction of the risk of acquiring a given disease or disorder.

25 In certain embodiments, the invention provides methods for treating or preventing diseases or disorders comprising administration of a modified PAI-1 molecule in combination with other treatments.

The most notable property of cancer is its ability to invade and metastasize. Proteolytic activity driven by urokinase plasminogen activator (uPA) or other proteolytic enzymes, such as metalloproteinases, cysteine and serine proteinases are able to degrade the 30 extracellular matrix. Highly metastatic cells synthesize various classes of degenerative enzymes and release them at higher concentrations or activities than their normal

angiogenesis or inhibition of other functions mediated or influenced by PAI-1, uPA, tPA, including but not limited to cell proliferation, primary and metastatic neoplastic diseases, e.g. cancer, cell attachment, cell migration, granulation tissue development, and/or inflammation. Accordingly, the invention provides methods of treating, preventing, 5 managing or ameliorating cancer, particularly metastatic cancer by administration of modified PAI-1 molecules of the invention.

Cancers and related disorders that can be treated or prevented by methods and compositions of the present invention include but are not limited to the following:

Leukemias such as but not limited to, acute leukemia, acute lymphocytic leukemia, acute 10 myelocytic leukemias such as myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia leukemias and myelodysplastic syndrome, chronic leukemias such as but not limited to, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering 15 multiple myeloma, nonsecretory myeloma, osteosclerotic myeloma, plasma cell leukemia, solitary plasmacytoma and extramedullary plasmacytoma; Waldenström's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone and connective tissue sarcomas such as but not limited to bone sarcoma, osteosarcoma, chondrosarcoma, Ewing's sarcoma, 20 malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, synovial sarcoma; brain tumors such as but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendrogioma, nonglial tumor, acoustic neurinoma, 25 craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, primary brain lymphoma; breast cancer including but not limited to adenocarcinoma, lobular (small cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease, and inflammatory breast cancer; adrenal cancer such as but not limited to pheochromocytom and adrenocortical 30 carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer such as but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers such as but limited to Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipius; eye cancers such as but not

review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia and Murphy et al., 1997, *Informed Decisions: The Complete Book of Cancer Diagnosis, Treatment, and Recovery*, Viking Penguin, Penguin Books U.S.A., Inc., United States of America)

5 Accordingly, the methods and compositions of the invention are also useful in the treatment or prevention of a variety of cancers or other abnormal proliferative diseases, including (but not limited to) the following: carcinoma, including that of the bladder, breast, colon, kidney, liver, lung, ovary, pancreas, stomach, cervix, thyroid and skin; including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage, including leukemia,  
10 acute lymphocytic leukemia, acute lymphoblastic leukemia, B-cell lymphoma, T-cell lymphoma, Berkett's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias and promyelocytic leukemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyoscarcoma; other tumors, including melanoma, seminoma, teratocarcinoma, neuroblastoma and glioma; tumors of the central  
15 and peripheral nervous system, including astrocytoma, neuroblastoma, glioma, and schwannomas; tumors of mesenchymal origin, including fibrosafcoma, rhabdomyoscarama, and osteosarcoma; and other tumors, including melanoma, xenoderma pegmentosum, keratoactanthoma, seminoma, thyroid follicular cancer and teratocarcinoma. It is also contemplated that cancers caused by aberrations in apoptosis would also be treated by the  
20 methods and compositions of the invention. Such cancers may include but not be limited to follicular lymphomas, carcinomas with p53 mutations, hormone dependent tumors of the breast, prostate and ovary, and precancerous lesions such as familial adenomatous polyposis, and myelodysplastic syndromes. In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative  
25 disorders, are treated or prevented in the ovary, bladder, breast, colon, lung, skin, pancreas, or uterus. In other specific embodiments, sarcoma, melanoma, or leukemia is treated or prevented.

30 In preferred embodiments, the methods and compositions of the invention are used for the treatment and/or prevention of breast, colon, ovarian, lung, and prostate cancers and melanoma and are provided below by example rather than by limitation.

In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias), or hyperproliferative disorders, are treated or prevented in the

In a specific embodiment, leukoplakia, a benign-appearing hyperplastic or dysplastic lesion of the epithelium, or Bowen's disease, a carcinoma *in situ*, are pre-neoplastic lesions indicative of the desirability of prophylactic intervention.

In another embodiment, fibrocystic disease (cystic hyperplasia, mammary dysplasia, 5 particularly adenosis (benign epithelial hyperplasia) is indicative of the desirability of prophylactic intervention.

In other embodiments, a patient which exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of the modified PAI-1 molecules of the invention: a chromosomal translocation associated 10 with a malignancy (e.g., the Philadelphia chromosome for chronic myelogenous leukemia, t(14;18) for follicular lymphoma, etc.), familial polyposis or Gardner's syndrome (possible forerunners of colon cancer), benign monoclonal gammopathy (a possible forerunner of multiple myeloma), and a first degree kinship with persons having a cancer or precancerous 15 disease showing a Mendelian (genetic) inheritance pattern (e.g., familial polyposis of the colon, Gardner's syndrome, hereditary exostosis, polyendocrine adenomatosis, medullary thyroid carcinoma with amyloid production and pheochromocytoma, Peutz-Jeghers syndrome, neurofibromatosis of Von Recklinghausen, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia, and 20 Bloom's syndrome; *see Robbins and Angell, 197, Basic Pathology, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 112-113* etc.)

In another specific embodiment, the modified PAI-1 molecules of the invention is administered to a human patient to prevent progression to ovary, breast, colon, lung, pancreatic, skin, prostate, gastrointestinal, B lymphocyte, T lymphocyte or uterine cancer, 25 melanoma or sarcoma.

The invention encompasses methods for treating or preventing a cancer or metastasis in a subject comprising in any order the steps of administering to the subject a modified PAI-1 molecule. In certain embodiments, the compositions and methods of the invention can be used to prevent, inhibit or reduce the growth or metastasis of cancerous 30 cells. In a specific embodiment, the administration of a modified PAI-1 molecule inhibits or reduces the growth or metastasis of cancerous cells by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at

(calreticulin fragment); ZD6126; ZD 6474; farnesyl transferase inhibitors (FTI); and bisphosphonates.

Additional examples of anti-cancer agents that can be used in the various embodiments of the invention, including pharmaceutical compositions and dosage forms and kits of the invention, include, but are not limited to: acivicin; aclarubicin; acodazole hydrochloride; acronine; adozelesin; aldesleukin; altretamine; ambomycin; ametantrone acetate; aminoglutethimide; amsacrine; anastrozole; anthramycin; asparaginase; asperlin; azacitidine; azetepa; azotomycin; batimastat; benzodepa; bicalutamide; bisantrene hydrochloride; bisnafide dimesylate; bizelesin; bleomycin sulfate; brequinar sodium; bropirimine; busulfan; cactinomycin; calusterone; caracemide; carbetimer; carboplatin; carmustine; carubicin hydrochloride; carzelesin; cedefingol; chlorambucil; cirolemycin; cisplatin; cladribine; crisnatol mesylate; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; daunorubicin hydrochloride; decitabine; dexormaplatin; dezaguanine; dezaguanine mesylate; diaziquone; docetaxel; doxorubicin; doxorubicin hydrochloride; droloxifene; droloxifene citrate; dromostanolone propionate; duazomycin; edatrexate; eflornithine hydrochloride; elsamitruclin; enloplatin; enpromate; epipropidine; epirubicin hydrochloride; erbulozole; esorubicin hydrochloride; estramustine; estramustine phosphate sodium; etanidazole; etoposide; etoposide phosphate; etoprine; fadrozole hydrochloride; fazarabine; fenretinide; floxuridine; fludarabine phosphate; fluorouracil; flurocitabine; fosquidone; fostriecin sodium; gemcitabine; gemcitabine hydrochloride; hydroxyurea; idarubicin hydrochloride; ifosfamide; ilmofosine; interleukin II (including recombinant interleukin II, or rIL2), interferon alfa-2a; interferon alfa-2b; interferon alfa-n1 ; interferon alfa-n3; interferon beta-I a; interferon gamma-I b; iproplatin; irinotecan hydrochloride; lanreotide acetate; letrozole; leuprolide acetate; liarozole hydrochloride; lometrexol sodium; lomustine; losoxantrone hydrochloride; masoprocol; maytansine; mechlorethamine hydrochloride; megestrol acetate; melengestrol acetate; melphalan; menogaril; mercaptopurine; methotrexate; methotrexate sodium; metoprine; meturedepa; mitindomide; mitocarcin; mitocromin; mitogillin; mitomalcin; mitomycin; mitosper; mitotane; mitoxantrone hydrochloride; mycophenolic acid; nocodazole; nogalamycin; ormaplatin; oxisuran; paclitaxel; pegaspargase; peliomycin; pentamustine; peplomycin sulfate; perfosfamide; pipobroman; pipsulfan; piroxantrone hydrochloride; plicamycin; plomestane; porfimer sodium; porfiromycin; prednimustine; procarbazine hydrochloride; puromycin; puromycin hydrochloride; pyrazofurin; riboprime; rogletimide; safingol; safingol hydrochloride; semustine; simtrazene; sparfosate sodium; sparsomycin;

agonists; estrogen antagonists; etanidazole; etoposide phosphate; exemestane; fadrozole; fazarabine; fenretinide; filgrastim; finasteride; flavopiridol; flezelastine; fluasterone; fludarabine; fluorodaunorubicin hydrochloride; forfenimex; formestane; fostriecin; fotemustine; gadolinium texaphyrin; gallium nitrate; galocitabine; ganirelix; gelatinase inhibitors; gemcitabine; glutathione inhibitors; hepsulfam; heregulin; hexamethylene bisacetamide; hypericin; ibandronic acid; idarubicin; idoxifene; idramantone; ilmofosine; ilomastat; imidazoacridones; imiquimod; immunostimulant peptides; insulin-like growth factor-1 receptor inhibitor; interferon agonists; interferons; interleukins; iobenguane; iododoxorubicin; ipomeanol, 4-; iroplact; irsogladine; isobengazole; isohomohalicondrin B; itasetron; jasplakinolide; kahalalide F; lamellarin-N triacetate; lanreotide; leinamycin; lenograstim; lentinan sulfate; leptolstatin; letrozole; leukemia inhibiting factor; leukocyte alpha interferon; leuprolide+estrogen+progesterone; leuprorelin; levamisole; liarozole; linear polyamine analogue; lipophilic disaccharide peptide; lipophilic platinum compounds; lissoclinamide 7; lobaplatin; lombricine; lometrexol; lonidamine; losoxantrone; lovastatin; loxoribine; lurtotecan; lutetium texaphyrin; lysofylline; lytic peptides; maitansine; mannostatin A; marimastat; masoprolol; maspin; matrilysin inhibitors; matrix metalloproteinase inhibitors; menogaril; merbarone; meterelin; methioninase; metoclopramide; MIF inhibitor; mifepristone; miltefosine; mirimostim; mismatched double stranded RNA; mitoguazone; mitolactol; mitomycin analogues; mitonafide; mitotoxin fibroblast growth factor-saporin; mitoxantrone; mofarotene; molgramostim; monoclonal antibody, human chorionic gonadotrophin; monophosphoryl lipid A+myobacterium cell wall sk; mopidamol; multiple drug resistance gene inhibitor; multiple tumor suppressor 1-based therapy; mustard anticancer agent; mycaperoxide B; mycobacterial cell wall extract; myriaporone; N-acetyl-dinaline; N-substituted benzamides; nafarelin; nagrestip; naloxone+pentazocine; napavine; naphterpin; nartograstim; nedaplatin; nemorubicin; neridronic acid; neutral endopeptidase; nilutamide; nisamycin; nitric oxide modulators; nitroxide antioxidant; nitrullyn; O6-benzylguanine; octreotide; okicenone; oligonucleotides; onapristone; ondansetron; ondansetron; oracin; oral cytokine inducer; ormaplatin; osaterone; oxaliplatin; oxaunomycin; paclitaxel; paclitaxel analogues; paclitaxel derivatives; palauamine; palmitoylrhizoxin; pamidronic acid; panaxytriol; panomifene; parabactin; pazelliptine; pegaspargase; peldesine; pentosan polysulfate sodium; pentostatin; pentozole; perflubron; perfosfamide; perillyl alcohol; phenazinomycin; phenylacetate; phosphatase inhibitors; picibanil; pilocarpine hydrochloride; pirarubicin; piritrexim; placetin A; placetin B; plasminogen activator inhibitor; platinum complex; platinum

to, cytosine arabinoside, taxoids (e.g., paclitaxel, docetaxel), anti-tubulin agents (e.g., paclitaxel, docetaxel, epothilone B, or its analogues), macrolides (e.g., rhizoxin) cisplatin, carboplatin, adriamycin, tenoposide, mitozantron, discodermolide, eleutherobine, 2-chlorodeoxyadenosine, alkylating agents (e.g., cyclophosphamide, mechlorethamine, 5 thioepa, chlorambucil, melphalan, carmustine (BSNU), lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin, thio-tepa), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, anthramycin), antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, flavopiridol, 5-fluorouracil, 10 fludarabine, gemcitabine, dacarbazine, temozolamide), asparaginase, *Bacillus Calmette Guerin*, diphtheria toxin, hexamethylmelamine, hydroxyurea, LYSODREN®, nucleoside analogues, plant alkaloids (e.g., Taxol, paclitaxel, camptothecin, topotecan, irinotecan (CAMPTOSAR, CPT-11), vincristine, vinca alkyloids such as vinblastine), podophyllotoxin (including derivatives such as epipodophyllotoxin, VP-16 (etoposide), 15 VM-26 (teniposide)), cytochalasin B, colchicine, gramicidin D, ethidium bromide, emetine, mitomycin, procarbazine, mechlorethamine, anthracyclines (e.g., daunorubicin (formerly daunomycin), doxorubicin, doxorubicin liposomal), dihydroxyanthracindione, mitoxantrone, mithramycin, actinomycin D, procaine, tetracaine, lidocaine, propranolol, puromycin, anti-mitotic agents, abrin, ricin A, pseudomonas exotoxin, nerve growth factor, 20 platelet derived growth factor, tissue plasminogen activator, aldesleukin, allutamine, anastrozole, bicalutamide, biaomycin, busulfan, capecitabine, carboplatin, chlorabusil, cladribine, cytarabine, daclinomycin, estramustine, floxuride, gancitabine, gosereine, idarubicin, itosfamide, lauprolide acetate, levamisole, lomusline, mechlorethamine, magestrol, acetate, mercaptopurino, mesna, mitolanc, pegaspargase, pentosatin, picamycin, 25 riuximab, campath-1, straplozocin, thioguanine, tretinoin, vinorelbine, or any fragments, family members, or derivatives thereof, including pharmaceutically acceptable salts thereof.

In other embodiments, the method for the treatment of cancers further comprises administration of pharmaceutical compositions comprising 5-fluorouracil, cisplatin, docetaxel, doxorubicin, Herceptin®, gemcitabine (Seidman, 2001, Oncology 15:11-14), IL-30 2, paclitaxel, and/or VP-16 (etoposide). In another embodiment, pharmaceutical compositions comprises modified PAI-1 molecules of the present invention conjugated with the above agents.

interferon- $\beta$ , macrophage inflammatory proteins, granulocyte monocyte colony stimulating factor, interleukins (including, but not limited to, interleukin-1, interleukin-2, interleukin-6, interleukin-12, interleukin-15, interleukin-18), OX40, CD27, CD30, CD40 or CD137 ligands, Fas-Fas ligand, 4-1BBL, endothelial monocyte activating protein or any fragments, 5 family members, or derivatives thereof, including pharmaceutically acceptable salts thereof.

In yet another embodiment, the treatment method further comprises hormonal treatment. Hormonal therapeutic treatments comprise hormonal agonists, hormonal antagonists (e.g., flutamide, tamoxifen, leuprolide acetate (LUPRON™), LH-RH antagonists), inhibitors of hormone biosynthesis and processing, steroids (e.g., 10 dexamethasone, retinoids, betamethasone, cortisol, cortisone, prednisone, dehydrotestosterone, glucocorticoids, mineralocorticoids, estrogen, testosterone, progestins), antigestagens (e.g., mifepristone, onapristone), and antiandrogens (e.g., cyproterone acetate).

The methods of the present invention may be used for the inhibition of angiogenesis 15 or inhibition of other functions mediated or influenced by PAI-1, uPA, or tPA, including but not limited to cell proliferation, primary and metastatic neoplastic diseases, cell attachment, cell migration, granulation tissue development, and/or autoimmune disorders or inflammatory diseases.

Examples of autoimmune disorders include, but are not limited to, alopecia areata, 20 ankylosing spondylitis, antiphospholipid syndrome, autoimmune Addison's disease, autoimmune diseases of the adrenal gland, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis and orchitis, autoimmune thrombocytopenia, Behcet's disease, bullous pemphigoid, cardiomyopathy, celiac sprue-dermatitis, chronic fatigue immune dysfunction syndrome (CFIDS), chronic inflammatory demyelinating 25 polyneuropathy, Churg-Strauss syndrome, cicatrical pemphigoid, CREST syndrome, cold agglutinin disease, Crohn's disease, discoid lupus, essential mixed cryoglobulinemia, fibromyalgia-fibromyositis, glomerulonephritis, Graves' disease, Guillain-Barre, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), IgA neuropathy, juvenile arthritis, lichen planus, lupus erthematosus, 30 Ménière's disease, mixed connective tissue disease, multiple sclerosis, type 1 or immune-mediated diabetes mellitus, myasthenia gravis, pemphigus vulgaris, pernicious anemia, polyarteritis nodosa, polychondritis, polyglandular syndromes, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis,

modulators. Examples of T cell receptor modulators include, but are not limited to, anti-T cell receptor antibodies (e.g., anti-CD4 monoclonal antibodies, anti-CD3 monoclonal antibodies, anti-CD8 monoclonal antibodies, anti-CD40 ligand monoclonal antibodies, anti-CD2 monoclonal antibodies) and CTLA4-immunoglobulin. Examples of cytokine receptor modulators include, but are not limited to, soluble cytokine receptors (e.g., the extracellular domain of a TNF- $\alpha$  receptor or a fragment thereof, the extracellular domain of an IL-1 $\beta$  receptor or a fragment thereof, and the extracellular domain of an IL-6 receptor or a fragment thereof), cytokines or fragments thereof (e.g., interleukin (IL)-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-15, TNF- $\alpha$ , TNF- $\beta$ , interferon (IFN)- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and GM-CSF), anti-cytokine receptor antibodies (e.g., anti-IL-2 receptor antibodies, anti-IL-4 receptor antibodies, anti-IL-6 receptor antibodies, anti-IL-10 receptor antibodies, and anti-IL-12 receptor antibodies), anti-cytokine antibodies (e.g., anti-IFN receptor antibodies, anti-TNF- $\alpha$  antibodies, anti-IL-1 $\beta$  antibodies, anti-IL-6 antibodies, and anti-IL-12 antibodies).

In a specific embodiment, the present invention provides a method for preventing, treating, managing or ameliorating an autoimmune or inflammatory disorder or one or more symptoms thereof, said method comprising administering to a subject in need thereof a prophylactically or therapeutically effective amount of modified PAI-1 molecules and a prophylactically or therapeutically effective amount of one or more immunomodulatory agents.

Anti-inflammatory agents have exhibited success in treatment of inflammatory and autoimmune disorders and are now a common and a standard treatment for such disorders. Any anti-inflammatory agent well-known to one of skill in the art can be used in the compositions and methods of the invention. Non-limiting examples of anti-inflammatory agents include non-steroidal anti-inflammatory drugs (NSAIDs), steroid anti-inflammatory drugs, beta-agonists, anticholinergic agents, and methyl xanthines. Examples of NSAIDs include, but are not limited to, aspirin, ibuprofen, celecoxib (CELEBREX<sup>TM</sup>), diclofenac (VOLTAREN<sup>TM</sup>), etodolac (LODINE<sup>TM</sup>), fenoprofen (NALFON<sup>TM</sup>), indomethacin (INDOCIN<sup>TM</sup>), ketorolac (TORADOL<sup>TM</sup>), oxaprozin (DAYPRO<sup>TM</sup>), nabumentone (RELAFEN<sup>TM</sup>), sulindac (CLINORIL<sup>TM</sup>), tolmentin (TOLECTIN<sup>TM</sup>), rofecoxib (VIOXX<sup>TM</sup>), naproxen (ALEVET<sup>TM</sup>, NAPROSYNT<sup>TM</sup>), ketoprofen (ACTRON<sup>TM</sup>) and nabumetone (RELAFEN<sup>TM</sup>). Such NSAIDs function by inhibiting a cyclooxygenase enzyme (e.g., COX-1 and/or COX-2). Examples of steroid anti-inflammatory drugs

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector or a delivery complex, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* 5 or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the modified PAI-1 protein and functionally active fragments, derivatives and analogs thereof. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression 10 vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in biopolymers (*e.g.*, poly- $\beta$ -1->4-N-acetylglucosamine polysaccharide; see 15 U.S. Patent No. 5,635,493), encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), etc. In another embodiment, a nucleic acid-ligand 20 complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 25 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Young). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses 30 are especially attractive vehicles for delivering genes to respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, *Current*

sequence, and the production of its protein product for functional analysis and/or therapeutic or diagnostic use, as described *infra*.

Alternatives to isolating the coding regions for the PAI-1 protein include, but are not limited to, chemically synthesizing the gene sequence itself from the published sequence.

- 5 Other methods are possible and within the scope of the invention. The above-methods are not meant to limit the following general description of methods by which modified PAI-1 protein may be obtained.

The modified PAI-1 molecules may be derived from other PAI-1 homologs. These PAI-1 homologs may be obtained by screening genomic libraries from other animals. A

- 10 PAI-1 analog preferably exhibits at least about 80% overall similarity at the amino acid level to the amino acid sequence depicted in Figure 2, more preferably exhibits at least about 85-90% overall similarity to the amino acid sequence in Figure 2 and most preferably exhibits at least about 95% overall similarity to the amino acid sequence in Figure 2. Such nucleic acid molecule that hybridizes to another nucleic acid consisting of the complement  
15 of the DNA sequences that encode the amino acid sequence shown in Figure 2 under moderately or low stringent conditions, e.g., hybridization to filter-bound DNA in 6X SSC at 45°C, and washing in 2xSSC at 50°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at page 2.10.3).

- 20 The modified PAI-1 molecules may also be derived from other naturally occurring variants of PAI-1, and degenerate variants thereof. A PAI-1 analog preferably exhibits at least about 80% overall similarity at the nucleotide level to the nucleic acid sequence depicted in Figure 1, more preferably exhibits at least about 85-90% overall similarity to the nucleic acid sequence in Figure 1 and most preferably exhibits at least about 95% overall  
25 similarity to the nucleic acid sequence in Figure 1. Such nucleic acid molecule that hybridizes to another nucleic acid consisting of the complement of the DNA sequences that encode the amino acid sequence shown in Figure 2 under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO<sub>4</sub>, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds.,  
30 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at page 2.10.3). The degree of similarity can be determined by analyzing sequence data using a computer algorithm, such as those used by the BLAST computer program.

the protein can be modified by any of numerous strategies known in the art (Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of a modified PAI-1 molecule, care should be taken to ensure that the modified gene remains within the same translational reading frame, uninterrupted by translational stop signals, in the gene region where the PAI-1 molecule is encoded.

Additionally, the nucleic acid sequence encoding the PAI-1 molecule can be mutated *in vitro* or *in vivo* to create variations in coding regions (e.g., amino acid substitutions), and/or to create and/or destroy translation, initiation, and/or termination sequences, and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), PCR-based overlap extension (Ho et al., 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar et al., 1990, Biotechniques, 8:404-407), etc. Mutations can be confirmed by double stranded dideoxy DNA sequencing.

In preferred embodiments, mutagenesis is used to substitute the codon for a particular amino acid residue with a codon for another amino acid residue, preferably an amino acid residue with a sulphydryl group.

Two or more amino acid residues within a PAI-1 protein can be substituted by another amino acid residue, most preferably an amino acid residue that can form a disulfide bridge. The amino acid to be introduced within the sequence may be selected from members of the same or different class to which the amino acid being substituted belongs. The nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. In a most preferred embodiment, the substitutions result in a modified PAI-1 molecule that has an increased half-life of the active form.

but are not limited to, the D-isomers of the common amino acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine,  
5 phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids, C $\alpha$ -methyl amino acids, N $\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In specific embodiments, the modified PAI-1 protein comprises a fusion protein that is produced by recombinant expression of a nucleic acid encoding a modified PAI-1 protein  
10 joined in-frame to the coding sequence for another protein, such as but not limited to toxins, such as ricin or diphtheria toxin. Such a fusion protein can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the fusion protein by methods commonly known in the art. Alternatively, such a fusion protein may be made  
15 by protein synthetic techniques, e.g., by use of a peptide synthesizer. Chimeric genes comprising portions of modified PAI-1 protein fused to any heterologous protein-encoding sequences may be constructed.

In other embodiments, the modified PAI-1 molecules are conjugated to a diagnostic or detectable agent. Such molecules can be useful for monitoring or prognosing the  
20 development or progression of a cancer as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can accomplished by coupling the modified PAI-1 molecules to detectable substances including, but not limited to various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic  
25 groups, such as but not limited to streptavidin/biotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not  
30 limited to iodine ( $^{131}\text{I}$ ,  $^{125}\text{I}$ ,  $^{123}\text{I}$ ,  $^{121}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulfur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{115}\text{In}$ ,  $^{113}\text{In}$ ,  $^{112}\text{In}$ ,  $^{111}\text{In}$ ), and technetium ( $^{99}\text{Tc}$ ), thallium ( $^{201}\text{Ti}$ ), gallium ( $^{68}\text{Ga}$ ,  $^{67}\text{Ga}$ ), palladium ( $^{103}\text{Pd}$ ), molybdenum ( $^{99}\text{Mo}$ ), xenon ( $^{133}\text{Xe}$ ), fluorine ( $^{18}\text{F}$ ),  $^{153}\text{Sm}$ ,  $^{177}\text{Lu}$ ,  $^{159}\text{Gd}$ ,  $^{149}\text{Pm}$ ,  $^{140}\text{La}$ ,  $^{175}\text{Yb}$ ,  $^{166}\text{Ho}$ ,  $^{90}\text{Y}$ ,  $^{47}\text{Sc}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{142}\text{Pr}$ ,  $^{105}\text{Rh}$ ,  $^{97}\text{Ru}$ ,  $^{68}\text{Ge}$ ,  $^{57}\text{Co}$ ,  $^{65}\text{Zn}$ ,  $^{85}\text{Sr}$ ,  $^{32}\text{P}$ ,

lymphokine (e.g., interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), and granulocyte-colony stimulating factor ("G-CSF")), or a growth factor (e.g., growth hormone ("GH")).

Moreover, the modified PAI-1 molecules may be conjugated to therapeutic moieties such as a radioactive metal ion, such as alpha-emitters such as  $^{213}\text{Bi}$  or macrocyclic chelators useful for conjugating radiometal ions, including but not limited to,  $^{131}\text{In}$ ,  $^{131}\text{Lu}$ ,  $^{131}\text{Y}$ ,  $^{131}\text{Ho}$ ,  $^{131}\text{Sm}$ , to polypeptides. In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo *et al.*, *Clin Cancer Res.* 4(10):2483-90 (1998); Peterson *et al.* *Bioconjug. Chem.* 10(4):553-7 (1999); and Zimmerman *et al.*, *Nucl. Med. Biol.* 26(8):943-50 (1999) each incorporated by reference in their entireties.

In specific embodiments, the modified PAI-1 molecule is conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytoidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

Further, the modified PAI-1 molecules may be conjugated to a therapeutic agent or drug moiety that modifies a given biological response. Therapeutic agents or drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or

hamster ovary cells (CHO) in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus (or promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter, the vaccinia virus 7.5K promoter) are effective  
5 expression systems; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus); plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid); microorganisms such as bacteria (e.g., *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid  
10 DNA or cosmid DNA expression vectors; yeast (e.g., *Saccharomyces Pichia*) containing recombinant yeast expression vectors. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific  
embodiments, a modified human PAI-1 protein coding region, or a sequence encoding a  
15 mutated and functionally active portion of the respective modified PAI-1 protein is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a modified PAI-1 gene consisting of appropriate transcriptional/translational control signals and the protein coding  
20 sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleotide sequence encoding a modified PAI-1 protein may be regulated by a second nucleotide sequence so that the modified PAI-1 protein is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a modified PAI-1 protein molecule may be  
25 controlled by any promoter/enhancer element known in the art. Promoters which may be used to include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-  
30 1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42).

In a preferred embodiment, the expression vector is pTYB12-PAI-1.

injection with the PAI-1 proteins and derivatives thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as 5 BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward modified PAI-1 protein molecules, any technique which provides for the production of antibody molecules by 10 continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 15 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal 20 Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-6855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule specific for the epitope together with genes from a human antibody 25 molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce specific single chain antibodies against PAI-1 proteins or fragments or derivatives thereof. An additional 30 embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

available in the art, such as BLAST, CHARMM release 21.2 for the Convex, and QUANTA v.3.3, (Molecular Simulations, Inc., York, United Kingdom).

Once a modified PAI-1 molecule is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The *in vitro* activities and *in vivo* biological functions of the foregoing may be evaluated using any suitable assay (including immunoassays as described *infra*).

Alternatively, once a modified PAI-1 protein produced by a recombinant host cell is identified, the amino acid sequence of the PAI-1 protein(s) can be determined by standard techniques for protein sequencing, *e.g.*, with an automated amino acid sequencer.

The modified PAI-1 protein sequence can be characterized by a hydrophilicity analysis (Hopp, T. and Woods, K., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the PAI-1 protein and the corresponding regions of the gene sequence which encode such regions.

Secondary structural analysis (Chou, P. and Fasman, G., 1974, Biochemistry 13:222) can also be done, to identify regions of the PAI-1 protein that assume specific secondary structures.

Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstrom, A., 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, *in* Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

The functional activity of modified PAI-1 protein molecules can be assayed by various methods known in the art.

For example, where one is assaying for the ability of a modified PAI-1 protein to bind or compete with wild-type PAI-1 proteins for binding to an antibody, uPA, or tPA, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as

from a subject over a period of time, for example but not limited to, immunoassays using anti-PAI-1 antibodies to measure the levels of the modified PAI-1 molecules in samples taken over a period of time after administration of the modified PAI-1 or detection of radiolabelled modified PAI-1 molecules in samples taken from a subject after 5 administration of the radiolabeled modified PAI-1 molecules.

Other methods will be known to the skilled artisan and are within the scope of the invention.

### 5.7. THERAPEUTIC USES

The invention provides for treatment, prophylaxis, management or amelioration of 10 one or more symptoms associated with the disease, disorder, or infection by administration of therapeutic compound (termed herein "Therapeutic") of the invention. Such Therapeutics include modified PAI-1 molecules having at least two amino acid substitutions at positions 31 and 97, 192 and 347, or 197 and 355 of the PAI-1 protein as depicted in Figure 2A (SEQ ID NO:2), preferably with two or more amino acid 15 substitutions at or near the  $\beta$ -sheet. More specifically, the two or more amino acid substitutions are at or near helix D,  $\beta$ -sheet b, and/or  $\beta$ -sheet t. The Therapeutics of the present invention include a modified PAI-1 molecule with mutations at amino acid positions located near or within the  $\beta$ -sheet so that one or more disulfide bridges may be formed that hold the A3 strand and the A5 strand of the  $\beta$ -sheet closer together, thus preventing the insertion of the A4 strand between the A3 strand and the A5 strand of the  $\beta$ -sheet. The Therapeutics of the present invention also include a modified PAI-1 protein in which two or more amino acid residues that do not contain a sulphydryl group have been replaced with amino acid residues that contain sulphydryl groups such as, but not limited to, 20 cysteine residues, that form one or more intrachain disulfide bonds, in which the active cysteine residues, that form one or more intrachain disulfide bonds, in which the active 25 form has a much longer *in vivo* half-life than a PAI-1 protein, such as a wild-type PAI-1 protein. Modified PAI-1 molecule comprises amino acid sequence in which two or more amino acid residues other than a cysteine residue or methionine residue are substituted with cysteine residues or methionine residues. The number of amino acid residues that may be substituted in a modified PAI-1 being 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 and less 30 than 20, more than 20, preferably, an even number of amino acid residues are substituted.

According to the invention, the modified PAI-1 molecules have substitutions, deletions or insertions, of two, four, six, or more amino acid residues in the wild type protein.

The Therapeutics can also be administered to an animal, preferably a mammal, and most preferably a human, to treat, prevent or ameliorate one or more symptoms associated with the disease, disorder, or infection related to abnormal angiogenesis, inflammation, or cancer.

- 5       The present invention provides Therapeutics for the treatment, prophylaxis, management or amelioration of one or more symptoms associated with the disease, disorder, or infection. These diseases, disorders, or infection manifest as cell proliferation, hyper-angiogenic diseases, psoriasis or inflammatory diseases. Disorders in which PAI-1 is absent or decreased relative to normal or desired levels are treated, prevented, managed, or  
10      ameliorated by administration of a modified PAI-1 molecule of the invention. Also, disorders that are related to increased uPA, or tPA relative to normal levels may also be treated, prevented, managed, or ameliorated by the methods of the invention.

- The absence of decreased level in PAI-1 protein or function can be readily detected, e.g., by obtaining a patient tissue sample (e.g., from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, structure and/or activity of the expressed RNA or protein of PAI-  
15      1. Many methods standard in the art can be thus employed, including but not limited to immunoassays to detect and/or visualize PAI-1 protein (e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect PAI-1  
20      expression by detecting and/or visualizing PAI-1 mRNA (e.g., Northern assays, dot blots, *in situ* hybridization, etc.), etc.

In specific embodiments, Therapeutics of the invention are used to treat cancer. The modified PAI-1 molecules are useful in decreasing invasion and metastatic.

- 25      In other specific embodiments, the modified PAI-1 of the invention can be used for targeted delivery of toxins such as, but not limited to, ricin, diphtheria toxin, etc.

#### 5.8. PHARMACEUTICAL COMPOSITIONS

- The invention provides methods of treatment, prophylaxis, management, or amelioration by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is  
30      preferably an animal, including but not limited to animals such as cows, pigs, horses,

porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

In another embodiment, the modified PAI-1 protein molecules can be delivered in a vesicle, in particular a liposome (see Langer, 1990, Science 249:1527-1533 (1990); Treat et al., in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the modified PAI-1 protein molecules can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980, *Surgery* 88:507; Saudek et al., 1989, *N. Engl. J. Med.* 321:574). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61; see also Levy et al., 1985, *Science* 228:190; During et al., 1989, *Ann. Neurol.* 25:351; Howard et al., 1989, *J. Neurosurg.* 71:105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in *Medical Applications of Controlled Release*, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer, 1990, *Science* 249:1527-1533 (1990). Other method of delivery of the therapeutics of the present invention may be used for example, as described in United States Patent No. 5,679,350, which is incorporated by reference in its entirety.

In a specific embodiment, a nucleic acid encoding modified PAI-1 protein molecules can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biostatic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:1864-1868),

sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a  
5 hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

10 The modified PAI-1 protein molecules of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-  
15 ethylamino ethanol, histidine, procaine, etc.

The amount of the modified PAI-1 protein molecules of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays and animal models may optionally be employed to help identify  
20 optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

In specific embodiments, the Therapeutics of the invention are administered  
25 intramuscularly. Suitable dosage ranges for the intramuscular administration are generally about 10 µg to 1 mg per dose, preferably about 10 µg to 100 µg per dose. In one embodiment, the Therapeutic is administered in two doses, where the second dose is administered 24 hours after the first dose; in another embodiment, the Therapeutic is administered in three doses, with one dose being administered on days 1, 4 and 7 of a 7 day  
30 regimen.

Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

The anti-cancer activity of the therapies used in accordance with the present invention also can be determined by using various experimental animal models of such as cancer animal models such as scid mouse model or nude mice with human tumor grafts known in the art and described in Yamanaka, 2001, Microbiol Immunol 2001;45(7):507-14.

- 5 For angiogenesis and tumor mouse model that are used in the present invention are discussed in Section 6.12.7 below.

The protocols and compositions of the invention are preferably tested *in vitro*, and then *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a

- 10 specific therapeutic protocol is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a protocol, and the effect of such protocol upon the tissue sample is observed. A lower level of proliferation or survival of the contacted cells indicates that the Therapeutic is effective to treat the condition in the patient. Alternatively, instead of culturing cells from a patient,  
15 Protocols may be screened using cells of a tumor or malignant cell line. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring  $^3\text{H}$ -thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (e.g., fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining,  
20 differentiation can be assessed visually based on changes in morphology, etc.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to in rats, mice, chicken, cows, monkeys, rabbits, etc.

- 25 The principle animal models for cancer known in the art and widely used include mice:, all described in Hann et al., 2001, Curr Opin Cell Biol 2001 Dec;13(6):778-84, which is incorporated herein by reference in its entirety.

Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic utility of the combinatorial therapies disclosed herein for treatment or prevention of cancer.

- 30 Efficacy in treating inflammatory disorders may be demonstrated by detecting the ability of the modified PAI-1 molecules of the present invention, or a composition of the

One liter of fresh LB broth medium containing ampicillin was inoculated with freshly grown culture and was incubated at 37°C, until the OD<sub>600</sub> of the cell culture reached 0.6. The expression of the 1B-14 mutant of human PAI-1 was stimulated by addition of IPTG to a final concentration of 0.5 mM. Next, cells were spun down, the cell pellet was 5 washed with 50 ml ice cold Cell Lysis Buffer (20 mM NA-HEPES, 500 mM Nalco, 1mM EDTA, 20 µM PMSF, 5 mM MgCl<sub>2</sub>, 10 µ/ml protease-free Dnase; pH = 8.00). After washing, cells were resuspended in 30 ml of Cell Lysis Buffer and broken down in a French press. The cell debris was removed by centrifugation and clear crude cell extract was transferred to a new tube and stored in the freezer at -20°C.

PAI-1 1B14 mutant was isolated on intein binding column. In this protocol, 30 ml of chitin bead resin was equilibrated with 10 bed volumes of Column Buffer (20 mM HEPES, 500 mM Nalco, 1 mM EDTA; pH – 8.00). Crude cell extract from 1 liter of cell culture was slowly applied onto chitin bead column at a 0.5 ml/min flow rate. In the next step, the column was extensively washed with 20 bed volumes of column buffer at 1.0 15 ml/min flow rate to remove unbound proteins. Next, the column was fast flushed with 3 bed volumes of Cleavage Buffer (20 mM HEPES, 500 mM Nalco, 1 mM EDTA, 50 mM DTT; pH = 8.00) at 2.0 ml/min and incubated for 40 h at 4 EC to stimulate on-column cleavage. The released from intein purification tag PAI-1 1B14 mutant was eluted from the column with Column Buffer. The eluted PAI-1 was dialyzed overnight against PBS Buffer 20 (120 mM Nalco, 2.7 mM KCl, 10 mM Na<sub>3</sub>PO<sub>4</sub>; pH 7.40) and concentrated. The purity of PAI-1 1B14 mutant was estimated as +95% as tested by SDS-PAGE. Yield was approximately 15-20 mg/l of cell culture. Activity of PAI-1 was verified by amidolytic assay.

## 6.2. Modified PAI-1 molecules

A total of seven cysteine mutants have been created via point mutation (two, four, and six point mutations) generating possible sites for disulfide bridge formation at the top and bottom parts of A3 and A5, within the helix D region, or by a combination thereof. Modified PAI-1 was expressed using a bacterial expression vector, pTYB12, producing a fused PAI-1/intein tag. The modified forms of PAI-1 containing the chitin binding intein 25 tag were then purified using affinity chromatography wherein the intein tag is cleaved leaving modified PAI-1 protein. Approximate protein yield was 1-5 mg/L of cell culture. The cysteine mutations appear to have no detrimental effects on enzymatic activity but can increase the t<sub>1/2</sub> of modified PAI-1 when compared to wild-type PAI-1. Among the mutants 30

inhibitor levels (PAI) in human plasma samples, but it can be adapted for tPA and PAI determinations in most biological fluids (eg, lymph and synovial fluids) as well as cell culture supernatants and animal plasmas.

The SPECTROLYSE® tPA/PAI assay is based on the functional parabolic rate assay described by Ránby<sup>1</sup> and its adaptation to plasma samples as described Wiman<sup>2</sup>. It is a rapid and convenient assay utilizing a final mixture containing sample, plasminogen, fibrin and a plasmin substrate. tPA in the sample activates the plasminogen to plasmin. The plasmin cleaves the plasmin substrate generating a yellow colored solution. The absorbance of the solution measured at 405 nm is a quantitative measure of the IPA activity in the sample. Use of the unique fibrin stimulator DESAFIB® and the highly efficient plasmin substrate SPECTROZYME® PL together provide a highly sensitive, selective and single-tube assay.

Reagents: Human Glu-plasminogen, lyophilized; DESAFIB®-X: lyophilized; SPECTROZYME® PL: lyophilized; tPA Standard, 2-chain:lyophilized; TRIS Buffer; Acetate Buffer; Stop Solution. (contains SDS).

#### 6.4.1. Determination of tPA activity of blood plasma

Add 100 µL Acetate buffer to 200 µL freshly drawn blood. This acidification should be done within 15 seconds of collection to prevent IPA from complexing with PAI and causing artificially low tPA activity levels. Centrifuge the blood sample at 2000g for 5 minutes (within 15 minutes of acidification to avoid hemolysis). Pipette 100 µL of supernatant (acidified plasma) to a new tube and add 100 µL acetate buffer and 100 µL TRIS Buffer. Incubate at 37°C for 20 minutes (to destroy alpha-2-antiplasmin which interferes with the assay). SPECTROLYSE® tPA/PAI can be applied to previously frozen plasma samples providing they were acidified prior to freezing. When frozen at -80°C, tPA activity is stable for several months<sup>2</sup>. Any precipitate observed upon thawing appeals to have no effect on tPA activity. For Cell Culture Supernatants, follow the first step and filter the supernatant using a 0.22 micron low protein binding membrane to remove any cell debris.

Where  $H_c$  = average hematocrit of the blood sample. For example, if the  $H_c$  = 0.45, the dilution factor is 5.7. If you wish to report the results as tPA activity in whole blood, use  $H_c$  = 0, so that the dilution factor is 4.5.

#### 6.6. Expected tPA activity values

5       The tPA activity level in mammalian plasma varies greatly. The basal (resting) levels of tPA range from 0.0-0.04 IU/mL in normal patients and from 0.0- 0.85 IU/mL for DVT patients. Following venous occlusion, exercise stress, and desmopression infusion, tPA levels have been reported to range from 1.4 - 14 IU/mL for normal patients and 0.0.- 25 IU/mL for DVT patients. Wilman et al., 1985, J. Lab Clin Med 105:265-270. Owing to  
10      these wide ranges of reported valves, it is recommended that venous occlusion, exercise, and desmopression samples be measured using a 2 hour, 37°C incubation and that basal level samples be measured using a 20 hour, 37°C incubation.

#### 6.7. Determination of PAI levels in blood plasma

The SPECTROLYSE® tPA/PAI assay is suitable for determination of tPA inhibitor,  
15      PAI. The inhibitor level is determined as the difference between the amount of tPA activity added to the plasma sample and the amount of tPA activity remaining after a defined period of time. Wilman et al., 1984, J. Biol Chem 259:3644-3647. PAI analysis can be performed on citrated (STABILYTET™ tube), EDTA or heparinized plasma. Alternatively, acetate prepared plasma as described in Part I, SAMPLE COLLECTION AND PREPARATION,  
20      A. Plasma, Steps 1 and 2 on page 3 of this insert may be used. (Do not add additional Acetate arid TRIS Buffer as described In Step 3). Remember to record the dilution factor of the patient blood for later calculations.

#### 6.8. Preparation of standards for standard curve

Use the same standards as described in section 6.5. Dilute test sample 1:2 with  
25      TRIS Buffer. Add 100 µL of diluted test sample to 100 µL of (PA activity standard and incubate for 15 minutes at room temperature. This allows for the tPA and PAI to react and complex. As described earlier, a 10 IU/mL tPA standard will be used for example. Note the tPA activity on the vial label and the subsequent activity level of the stock solution generated. Add 250 µL Acetate buffer to the sample and incubate at 37°C for 15 minutes (to  
30      destroy alpha-2-anti-plasmin which would otherwise interfere with the assay). At this point, the sample contains a maximum of 2.22 IU/mL of tPA activity if no PAI were present in the plasma. Prepare a "0 PAI" as follows: Add 250 µL Acetate buffer, 100 µL test sample

$\mu$ L of buffer, 50  $\mu$ L of filtered sample (free of particulate and colored matter), or UK standards or controls, and 50  $\mu$ L of SPECTROZYME® UK. Mix and incubate 15 minutes at room temperature or 3 minutes at 37°C. Read the absorbance on a micro-plate reader (405 nm) or repeatedly during the following 10 minutes:

- 5 The lower limit of detection of this direct urokinase assay is approximately 20 IU/mL. Sensitivity is estimated to be 4-5 IU/mL of sample. A standard curve can be prepared using Products No. 124 or No. 125, (HMW and LMW urokinase activity standards, respectively).

### **6.11. Urokinase Activity Determination - Indirect Assay**

- 50mM Tris with 0.01% Tween 80 or 0.1% PEG 8000, pH 7.4. SPECTROZYME® PL at  
10 0.4 mM concentration (ADI Product No. 251). Add 6 mM EACA (6-aminohexanoic acid) and 0.1 mg/mL Bovine Plasminogen (ADI Product No. 416) to the buffer. Follow protocol listed above for the Direct Assay. The assay sensitivity is estimated to be 10-100 fold higher than the direct assay. The incubation time must be monitored as there is a definite risk of activation of pro-UK (scu-PA) by the plasmin formed. Urine samples exhibit absorbance at  
15 405 nm (approximately 0.100 OD for a 10% urine solution). Urine samples must be diluted with buffer prior to assay to reduce the background signal. Alternatively, measure this background absorbance and deduct the value from the sample readings.

### **6.12. Anti-angiogenic activity of PAI-1 with extended half-life**

#### **6.12.1. Sprout Formation Assay**

- 20 Sprout formation assay was performed using human endothelial cells to determine anti-angiogenic activity of PAI-1. These cells express high activity of uPA on the tip of the sprout when grown in fibrin gels. Inhibition of uPA will prevent sprout formation and will be a measure of anti-angiogenic activity on the human cell model.

#### **6.12.2. Preparation of human umbilical vein endothelial cells (HUVEC) aggregates**

- 25 HUVEC were grown to confluence in EGM-2 growth medium. Cells were trypsinized and seeded onto 0.5% agarose coated culture dishes. This procedure resulted in cells aggregate formation after 24 h of incubation at +37°C. The HUVEC aggregates were decanted under gravitational force by allowing the cells to stand for 30 min. at room  
30 temperature. The old-medium supernatant was decanted and HUVEC aggregates were suspended in 5 ml of fresh EGM-2 growth medium.

methylcellulose disks in all cases as shown in Fig 9. Additionally, angiogenesis was observed in the large avascular zones outside of areas covered by methylcellulose disk containing the inhibitor. This effect was observed for B428 and amiloride. In contrast, control CAMs implanted on the empty methylcellulose disks without inhibitors did not 5 develop avascular zones as determined by visual examination (Fig. 9A). As the positive control, methylcellulose disk containing VEGF was implanted and dense areas of newly formed vessels were developed. The percentage of area covered by blood vessels were presented in Figure 10.

#### 6.12.6. Image processing and analysis

10 The area below the dialysis bag and two non-treated areas were scanned and saved on computer disk as tif files. Color images were converted into black/white images, contrast was enhanced, and images were saved as 16-bitmap files using Paint Software (Microsoft Corporation, Redmond, VA). Black/white images were converted into false color (rainbow striped) images using Transform2 Software (Fortner, Sterling, VA). Finally, 15 the area of the blood vessels was calculated using T3D Software (Fortner, Sterling, VA). This method provides some quantification of angiogenesis.

#### 6.12.7. The animal model of tumor progression

50 SCID/BALB-c mice (males) were subcutaneously inoculated in the left rear flank with  $1.0 \times 10^6$  cells of LNCaP expressing undetectable amounts of u-PA. After 30 days 20 when the tumor was 3 mm in diameter, animals were divided into 6 groups. Control-1 group ( $n = 8$ ) composed of SCID-mice inoculated with LNCaP tumor cells. Control-2 group ( $n = 8$ ) composed of SCID-mice inoculated with LNCaP cells and receiving saline solution delivered via ALZET® osmotic pump. There were 4 treatment groups ( $n = 8$ , each) composed of SCID-mice inoculated with LNCaP tumor cells and receiving 1B-14 25 human PAI-1 mutant solution at concentrations of  $5.00 \times 10^{-10}$  M,  $2.50 \times 10^{-9}$  M,  $5.00 \times 10^{-9}$  M, and  $10.00 \times 10^{-9}$  M, respectively. The PAI-1 solution was delivered via an ALZET® osmotic pump.

A 200  $\mu$ L (model #2004) osmotic pump was used and the 1B-14 human PAI-1 mutant and saline solutions were delivered at a flow rate of 0.25  $\mu$ L/h. Tumor size in each 30 group was measured every 3 days over the 28-day course of the experiments and the volume was calculated using the following formula:

inhibitors decrease angiogenesis in the chicken embryo model. Swiercz et al., 1999, Oncology Reports 6:523-526.

Inhibitors of urokinase limit cancer growth by inhibiting angiogenesis. However, uPA inhibitors can act on cancer cells directly or prevent angiogenesis by an alternative  
5 mechanism not related to uPA inhibition.

The present invention is not to be limited in scope by the microorganism deposited or the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are  
10 intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties. Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

References:

- 15 Rånby et al., 1982, Thrombosis Research 27:743-749.  
Wiman et al., 1983, Clin. Chem Acta 127:279-288.

11. A method of producing a modified PAI-1 molecule said method comprising:  
(a) introducing into a cell a nucleic acid molecule encoding a modified PAI-1 molecule in  
which two or more amino acid residues that do not contain a sulphydryl group are each  
substituted by an amino residue that contains a sulphydryl group; (b) culturing the cell under  
5 conditions suitable for expression of the modified PAI.

12. The method of claim 11 wherein said two or more amino acid residues that  
do not contain a sulphydryl group are selected from among positions 31, 97, 192, 197, 347,  
and 355 of the amino acid sequence of the PAI-1 protein of SEQ ID NO:2.

13. The method of claim 11 wherein said two or more amino acid residues that  
10 do not contain a sulphydryl group are selected from one or more pairs of amino acid  
positions selected from the group consisting of 31 and 97, 192 and 347, and 197 and 355 of  
the amino acid sequence of the PAI-1 protein of SEQ ID NO:2.

14. A method of treating or preventing a disease or disorder related to aberrant  
angiogenesis in a subject in need thereof, said method comprising administering to a subject  
15 in which such treatment or prevention is desired an effective amount of the modified PAI-1  
molecule of claim 1.

15. A method of treating or preventing cancer in a subject suffering therefrom,  
said method comprising administering to a subject in which such treatment or prevention is  
desired an effective amount of the modified PAI-1 molecule of claim 1.

20 16. The method of claim 15 wherein said cancer is selected from the group  
consisting of breast cancer, colon cancer, ovarian cancer, lung cancer, prostate cancer,  
melanoma, leukemia, lung cancer, skin cancer, pancreatic cancer, bladder cancer, sarcoma,  
and uterine cancer.

25 17. A method of treating or preventing a cardiovascular disease or disorder in a  
subject, said method comprising administering to a subject in which such treatment or  
prevention is desired an effective amount of the modified PAI-1 molecule of claim 1.

18. The method of claim 17 wherein said disorder is hyperfibrinolysis,  
hemophilia, or vessel leakage syndrome.

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1 GAATTCCCTGCAGCTCAGCAGCCGCCAGAGCAGGACGAACCGCCAATCGCAAGGCACC  
 1-----+-----+-----+-----+-----+-----+ 60  
 CTTAAGGACGTGAGTCGTGGCGGGTCTCGTCTGCTTGGCGTTAGCGTTCCGTGG  
  
 61 TCTGAGAACCTCAGGATGCAGATGTCTCCAGCCCTCACCTGCCTAGTCCTGGGCC  
 61-----+-----+-----+-----+-----+-----+ 120  
 AGACTCTGAAAGTCCTACGTCTACAGAGGTCGGAGTGGACGGATCAGGACCCGGACCGG  
 aa M Q' M S P A L T C L V L G L A -  
 | Signal Peptide  
 CTTGTCTTGGTGAAGGGTCTGCTGTGCACCATCCCCATCCTACGTGGCCCACCTGGCC  
 121-----+-----+-----+-----+-----+-----+ 180  
 GAACAGAAACCACTTCCCAGACGACACGTGGTAGGGGTAGGATGCACCGGGTGGACCGG  
 aa L V F G E G S A | V K H P P S Y V A H L A 12  
 Start Mature Protein  
 TCAGACTTCGGGGTGAGGGTGTTCAGCAGGTGGCGAGGCTCCAAGGACCCGACCGTG  
 181-----+-----+-----+-----+-----+-----+ 240  
 AGTCTGAAAGCCCCACTCCCACAAAGTCGTCCACCGCGTCCGGAGGTTCTGGCGTTGCAC  
 aa S D F G V R V F Q Q V A Q A S K D R N V 32  
  
 GTTTTCTCACCTATGGGGTGGCCTCGGTGTTGGCATGCTCCAGCTGACAACAGGAGGA  
 241-----+-----+-----+-----+-----+-----+ 300  
 CAAAAGAGTGGGATAACCCCACCGGAGCCACRAACCGTAGGAGGTGACTGTTGTCCTCCT  
 aa V F S P Y G V A S V L A M L Q L T T G G 52  
  
 GAAACCCAGCAGCAGATTCAAGCAGCTATGGGATTCAAGATTGATGACAAGGGCATGGCC  
 301-----+-----+-----+-----+-----+-----+ 360  
 CTTTGGGTGTCGTCTAAGTTCGTGATACCCCTAAGTCTAACTACTGTTCCCGTACCGG  
 aa X T Q Q I Q A A M G F K I D D X G M A 72  
  
 CCCGCCCTCCGGCATCTGTACAAGGAGCTCATGGGGCATGGAACAAGGATGAGATCAGC  
 361-----+-----+-----+-----+-----+-----+ 420  
 GGGCGGGAGGCCGTAGACATGTTCTCGAGTACCCGGTACCTTGTCTACTCTAGTCG  
 aa P A L R H L Y K E L M G P W N K D E I S 92  
  
 ACCACAGACGCCATCTCGTCCAGCGGGATCTTAAGCTGGTCCAGGGCTTCATGCCAAC  
 421-----+-----+-----+-----+-----+-----+ 480  
 TGGTGTCTGGCTAGAACAGGTCGCCCTAGACTTCGACCGAGTCCCGAAGTACGGGGTG  
 aa T T D A I F V Q R D L K L V Q G F M P K 112  
 TTCTTCAGGCTGTTCCGGAGCACGGTCAAGCAAGTGGACTTTCAAGAGGTGGAGAGAGCC  
 481-----+-----+-----+-----+-----+-----+ 540  
 AAGAAGTCCGACAAGGCCCTGTCAGTTGTTACCTGAAAAGTCTCCACCTCTCTCGG  
 aa F F R L F R S T V K Q V D F S E V E R A 132  
  
 AGATTCAATCATCAATGACTGGGTGAAGACACACACAAAAGGTATGATCAGCAACTTGCTT  
 541-----+-----+-----+-----+-----+-----+ 600  
 TCTAAGTAGTAGTTACTGACCCACTTCTGTGTTCCATACTAGTCGTTAACGAA  
 aa R F I I N D W V K T H T K G M I S N L L 152

FIG. 1A

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601 GGGAAAGGAGCCGTGGACCAAGCTGACACGGCTGGTGTGGTGAATGCCCTACTTCAC  
 aa CCGTTTCCCGCACCTGGTCACTGTGCCAACGACCACTTACGGGAGATGAAGTTG  
 S K G A V D Q L T R L V L V N A L Y F N 172  
  
 661 GGCCAGTGGAAAGACTCCCTCCCCACTCCAGCACCCACGCCGCCTTCCACAAATCA  
 aa CCGGTCACCTTCTGAGGGAAAGGGCTGAGGTCTGGTGGCGGGAGAAGGTGTTAGT  
 G Q W X T P F P D S S T H R R L F H K S 192  
  
 721 GACGGCAGCACTGTCTGTGCCCATGATGGCTCAGACCAACAAGTTCAACTATACTGAG  
 aa CTGCCGTCGTGACAGAGACACGGGACTACCGAGTCTGGTTCAAGTTGATATGACTC  
 D G S T V S V P M M A Q T H K F N Y T E 212  
  
 781 TTCACCACGCCGATGCCATTACTACGACATCCTGGAACGCCCTACCAACGGGACACC  
 aa AAGTGGTGGGGCTACCGGTAATGATGCTGTAGGACCTGACGGGATGGTGCCCCCTGTGG  
 F T T P D G H X Y D I L E L P Y H G D T 232  
  
 841 CTCAGCATGGTCATTGCTGCCCTTATGAAAAAGAGGTGCCCTCTCTGCCCTACCAAC  
 aa GAGTCGTACAAGTAACGACGGGAATACTTTTCTCCACGGAGAGAGACGGGAGTGGTTG  
 L S M F I A A P Y E K E V P L S A L T N 252  
  
 901 ATTCTGAGTCCCAGCTCATCAGCCACTGGAAAGGCAACATGACCAGGCTGCCGCCTC  
 aa TAAGACTCACGGGTCGAGTAGTCGGTGACCTTCCGTTGACTGGTCCACGGGGAG  
 I L S A Q L I S H W X G N M T R L P R L 272  
  
 961 CTGGTTCTGCCAACGTTCTCCCTGGAGACTGAAGTCGACCTCAGGAACGCCCTAGAGAAC  
 aa GACCAAGACGGGTTCAAGAGGGACCTCTGACTTCAGCTGGAGTCCCTGGGGATCTCTG  
 L V L P K F S L E T E V D L R K P L E N 292  
  
 1021 CTGGGAATGACCGACATGTTCAGACAGTTCAGGCTGACTTCAGAGTCCTTCAGACCAA  
 aa GACCCCTTACTGGCTGTACAAGTCTGCAAGTCCGACTGAAGTGTCAAGAAAGTCTGGTT  
 L G M T D K F R Q A D F T S L S D Q 312  
  
 1081 GAGCCTCTCACGTCGCGCAGGGCTGCAGAAAGTGAAGATCGAGGTGAACGAGAGTGGC  
 aa CTCGGAGAGGTGCAGCGCTCCCGCACGTCTTCACTTCTAGCTCCACTTGCTCTCACCG  
 E P L H V A Q A L Q K V X I E V N R S G 332  
  
 1141 ACGGTGGCCCTCTCATCCACAGCTGTCAAGTCAGCCGCATGGCCCCCGAGGAGATC  
 aa TGCCACCGGAGGGTAGGTGTGACAGTATCAGAGTCGGGCGTACCGGGGGCTCTCTAG  
 T V A S S S T A V I V S A R M A P H E I 352  
  
 1201 ATCATGGACAGACCCCTCTTGTGGTCCGGCACACCCACAGGAACAGTCCTTTC  
 aa TAGTACCTGTCTGGGAAGGAGAACACCAAGGCCGTGTTGGGTGTCTGTCAAGGAAAAG  
 I M D R P F L F V V R H N P T G T V L F 372

FIG. 1B

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ATGGGCCAAGTGATGGAACCTGTACCCCTGGGGAAAGACGCCTCATCTGGGACAAAACGT  
 1261 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1320  
 TACCCGGTTCACTACCTTGGGACTGGGACCCCTTCTGCGGAAGTAGACCCCTTTGAC  
 M G Q V K E P \* 379

GAGATGCATGGGAAAGAAGAAAACCTCGAAGAAAAGAATTAGTGTAAATGACTCTTC  
 1321 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1380  
 CTCTACGTAGCCCTTCTTCTTGGAGGCTTCTTCTTAAATCACAAATTACTGAGAARG

TGAAGGAAGAGAAGACATTTGCCCTTTGTTAAAGATGGTAAACCAGATCTGCTCCAAG  
 1381 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1440  
 ACTTCCCTCTCTTGTAAACGGAAAACAATTCTACCATTGGTCTAGACAGAGGTTCA

ACCTTGGCCTCTCTTGGAGGACCTTGTAGTCACCTCCCTAGTCTCCACCTGAGACCC  
 1441 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1500  
 TGGAACCGGAGAGGAACCTCTGGAAATCCAGTTGAGGGATCAGAGGTGGACTCTGGGA

GGGAGAGAAGTTGAAGCACAACCTCCCTAACGGTCTCCAAACCAAGCGGTGACCCCTGCG  
 1501 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1560  
 CCCTCTCTCAAACCTCGTGTGAGGGAAATTCCAGAGGTTGGTCTGCCACTGCCGACGC

GGACCATCTGGGGCACCTGCTCCACCCGTCCTCTGCCACTCGGGTCTGCAGACCTGG  
 1561 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1620  
 CCTGGTAGACCCCCGTGGACGAAGGTGGGAGAGAGACGGGTGAGGCCAGACGTCTGGACC

TTCCCACGTAGGCCCTTGCAAGGATGGAACACTACGGGCTTACAGGAGCTTTGTGTGCCT  
 1621 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1680  
 AAAGGTGACTCCGGAAACGTCTACCTGTAGTGTGCCCCAATGTCTCGAAAACACACGGGA

GGTAGAAACTATTTCTGTTCCAGTCACATTGCCATCACTCTGTACTGCCCTGCCACCGCG  
 1681 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1740  
 CCATCTTGATAAAAGACAAGGTCAAGTGTAAACGGTAGTGTAGAACATGACGGACGGTGGCGC

GAGGAGGCTGGTGACAGGCCAAGGCCAGTGGAAAGAAACACCCCTTCATCTCAGAGTCCA  
 1741 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1800  
 CTCCTCCGACCACTGTCCGGTTCCGGTCCTTCTTGTGGAAAGTAGAGTCTCAGGT

CTGTGGCACTGGCACCCCTCCCCAGTACAGGGTGCTGCAGGTGGCAGAGTGAATGTCC  
 1801 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1860  
 GACACCGTGACCCGGTGGGGAGGGGTCACTGTCCCCACGACGTCCACCGTCTCACTTACAGG

CCCATCATGTGGCCAACTCTCTGGCTGGCATCTCCCTCCCCAGAAACAGTGTGCAT  
 1861 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1920  
 GGGTAGTACACCGGTTGAGAGGACCGGACCGGTAGAGGGAGGGGTCTTGTACACCGTA

GGGTTATTTGGAGTGTAGGTACTTGTAACTCATTAAGCAGAATTCTGCTTCCTTT  
 1921 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 1980  
 CCCAATAAAACCTCACATCCACTGAACAAATGAGTAACCTCGTCTAAAGACGAAGGAAAA

ATTTTTATAGGAATAGAGGAAGAAATGTCAGATGCGTGCCCAGCTCTCACCCCCCAATC  
 1981 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2040  
 TAAAAATATCCTTATCTCTTCTTACAGTCTACGCACGGTGTGAGAAGTGGGGGGTAG

FIG. 1C

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TCTTGGTGGGAGGGGTGTACCTAAATATTATCATATCCTGCCCTTGAGTGTGTTA  
 2041 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2100  
 AGAACCAACCCCTCCCCACATGGATTTATAAATAGTAGGAACGGAACTCACGAACAAT

GAGAGAAAAGAGAACTACTAAGGAAAATAATATTATTTAAACTCGCTCCTAGTGTTC  
 2101 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2160  
 CTCTCTTCTCTGTGATGATTCCCTTTATTATAATTAATTTGAGCGAGGATCACAAAGAAA

GTGGTCTGTGTACCGTATCTCAGGAAGTCCAGCCACTTGACTGGCACACACCCCTCCGG  
 2151 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2220  
 CACCAGACACAGTGGCATAGAGTCCTCAGGTGGTGAAC TGACCGTGTGTGGGAGGGCC

ACATCCAGCGTGACGGAGCCCACACTGCCACCTTGAGACCCCTCGCGCCCC  
 2221 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2280  
 TGTAGGTGCACTGCCCTGGGTGTGACGGTGGAACACCGCGGACTCTGGGAGCGCGGGGG

CCCGCGCCCCCGCGCCCCCTTTTCCCTTGATGGAAATTGACCATACAATTTCATCCT  
 2281 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2340  
 GGCGCGGGGGGGCGCGGGGAGAAAAAGGGGAACACTACCTTAACGGTATGTTAAGTAGGA

CCTTCAGGGGATCAAAGGACGGAGTGGGGGACAGAGACTCAGATGAGGACAGAGTGTT  
 2341 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2400  
 GGAAGTCCCCTAGTTTCCCTGCCTCACCCCCCTGTCTGTGAGTCTACTCCTGTCTCACCA

TTCCAATGTGTTCAATAGATTTAGGAGCAGAAATGCAAGGGCTGCATGACCTACCAGGA  
 2401 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2460  
 AAGGTTACACAAGTTATCTAAATCCTCGTCTTACGTTCCCCGACGTACTGGATGGTCT

CAGAACTTTCCCCAATTACAGGGTGACTCACAGCCGCATTGGTACTCACTTCATGTGT  
 2461 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2520  
 GTCTTGAAAGGGGTTAATGTCCCACTGAGTGTGCGCGTAACCACTGAGTGAAGTTACACA

CATTCCGGCTGCTGTGAGCAGTGGACACGTGAGGGGGGGTGGGTGAGAGAGACA  
 2521 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2580  
 GTAAAGGCCGACGACACACACTCGTCACCTGTGCACTCCCCCCCCACCCACTCTCTGT

GGCAGCTGGATTCAACTACCTTAGATAATATTCTGAAAACCTACCAAGCCAGAGGGTAG  
 2581 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2640  
 CCGTCGAGCCTAAGTTGATGGAATCTATTATAAGACTTTGGATGGTCGGTCTCCCATC

GGCACAAAGATGGATGTAATGCACTTGGGAGGCCAAGGCCGGAGGATTGCTTGAGCCCA  
 2641 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2700  
 CCGTGTCTACCTACATTACGTGAAACCCCTCCGGTCCGCCCTCTAACGAACCTCGGGT

GGAGTTCAAGACCAGCCTGGCAACATACCAAGACCCCCGTCTTTAAAAATATATA  
 2701 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2760  
 CCTCAAGTTCTGGTCGGACCCGGTGTATGGTTCTGGGGGAGAGAAATTATATATAT

TTTTAAATATACTTAAATATATATTTCTAATATCTTAAATATATATATATTTTAAAG  
 2761 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2820  
 AAAATTATATGAATTATATAAGATTATAGAAATTATATATATATAAAATTTC

ACCAATTATGGGAGAATTGCAACACAGATGTGAAATGAAATGTAATCTAACAGAGC  
 2821 -----+-----+-----+-----+-----+-----+-----+-----+-----+ 2876  
 TGGTTAAATACCCCTTAAACGTGTCTACACTTACATTAGATTATCTTCG

FIG. 1D

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MOMSPALTCLVLGLALVFGEGLSA  
Signal peptide

VHHPPSYVAHLASDFGVRFQQVAQASKDRNVVFSPYGVASVIALMLQLTTGGETQQQIQA  
AMGFKIDDKGMAPALRHYKELMGPWNKDEISTTDAIFVQRDLKLVQGEMPHFFRLFRST  
VKQVDFSEVERARFIINDWVKHTKGMISNLLGRGAVDQLTRLVLVNALYFNGQWKTPFP  
DSSTHRRLFHKSdgstvsvpmmaqtNKFNYTEFTTPDGHYYDILELPYHGDTLSMFIAAP  
YEKEVPLSALTNIQLISHWKGNMTRLPRLLVLPKFSLETEVDLRKPLENLGMTDMFR  
QFQADFTSLSDQEPLHVAQALQKVKIEVNESGTVASSSTAVIVSARMAPEEIIMDRPFLF  
VVRHNPTGTVLFMGQVMEP

FIG. 2A

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10                    20                    30                    40                    50                    60  
VHHPPSYVAHLASDFGVRVFQQVAQASKDRNVVFSPYGVASVLAMLQLTTGGETOQQIQA

70                    80                    90                    100                    110                    120  
AMGFKIDDKGMAPALRHLYKELMGPWNKDEISTTDAIFVQRDLKLVQGFMPHFFRLFRST

130                    140                    150                    160                    170                    180  
VKQVDFSEVERARFIINDWVKTHTKGMISNLLGKGAVDQLTRLVLVNALYFNGQWKTPFP

190                    200                    210                    220                    230                    240  
DSSTHRRLFHKSdgstvsvpmmaqtNKFNYTEFTTPDGHYYDILELPYHGDTLSMFIAAP

250                    260                    270                    280                    290                    300  
YEKEVPLSALTNIQLISHWKGNMTRLPRLLVLPKFSLETEVDLRKPLENLGMTDMFR

310                    320                    330                    340                    350                    360  
QFQADFTSLSDQEPLHVAQALQKVKIEVNESGTVASSSTAVIVSARMAPEEIIMDRPFLF

370  
VVRHNPTGTVLFMGQVMEP

FIG. 2B

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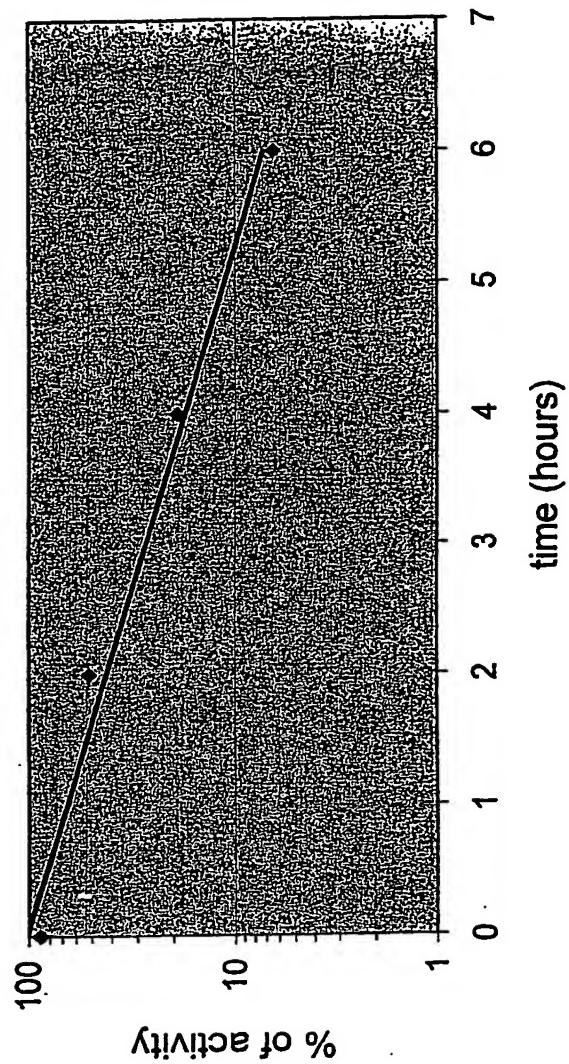


FIG. 3

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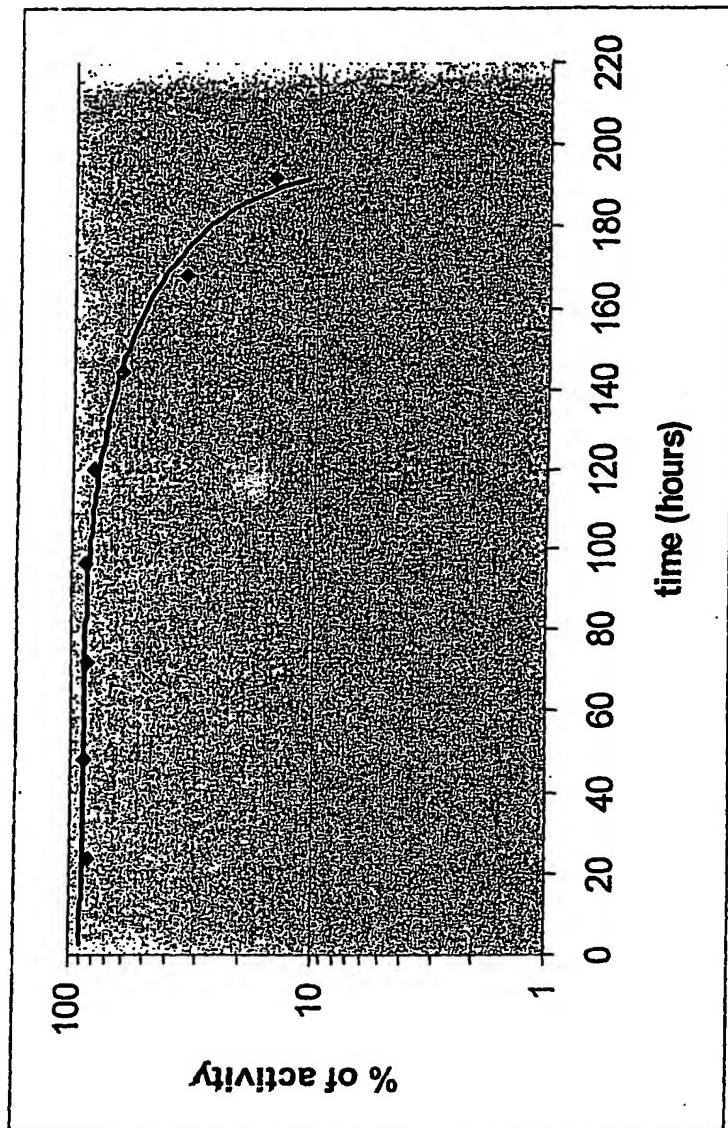


FIG. 4

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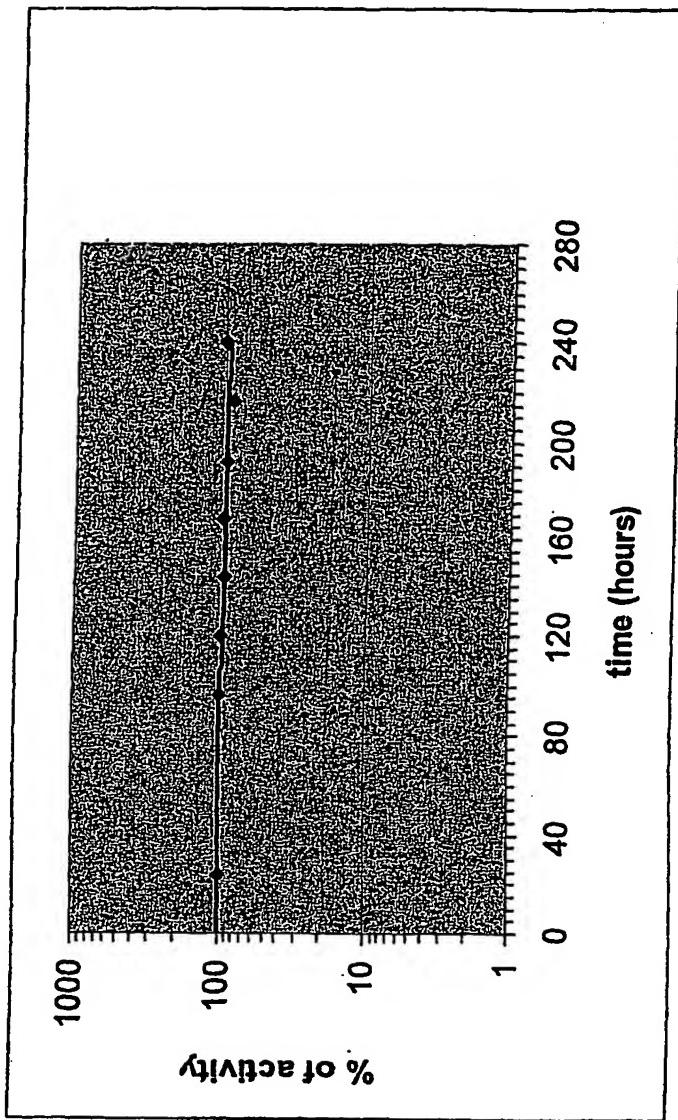


FIG. 5

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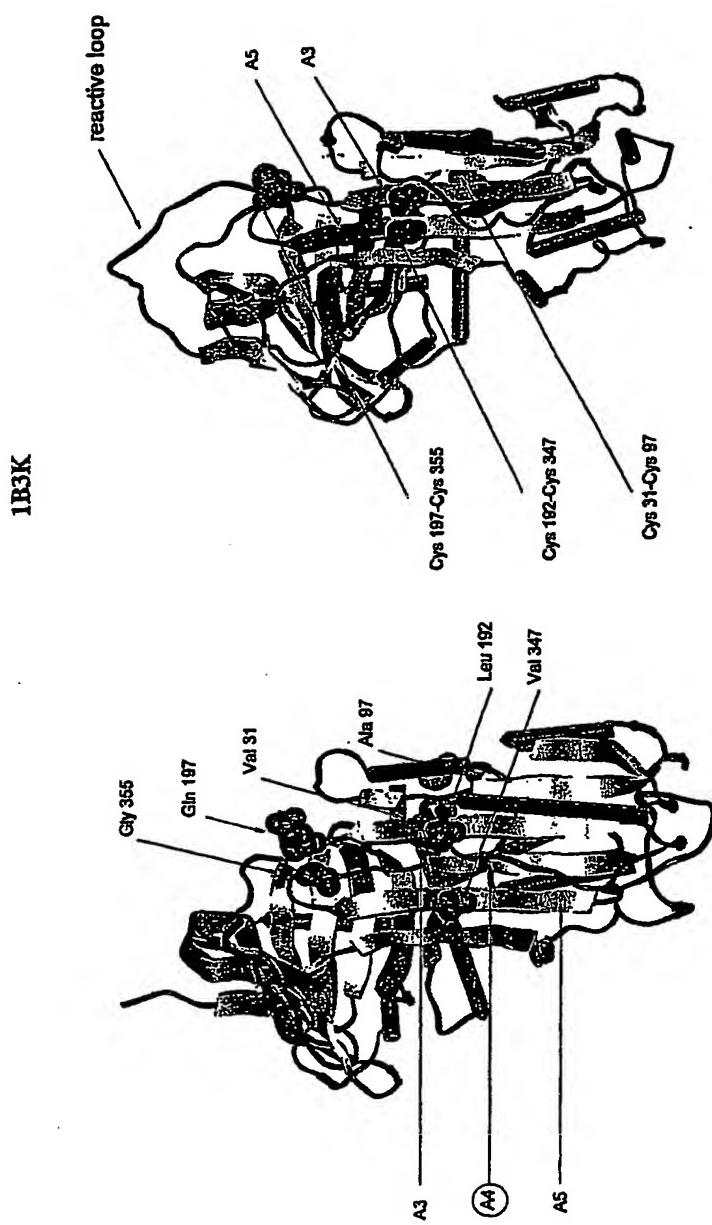


FIG. 6

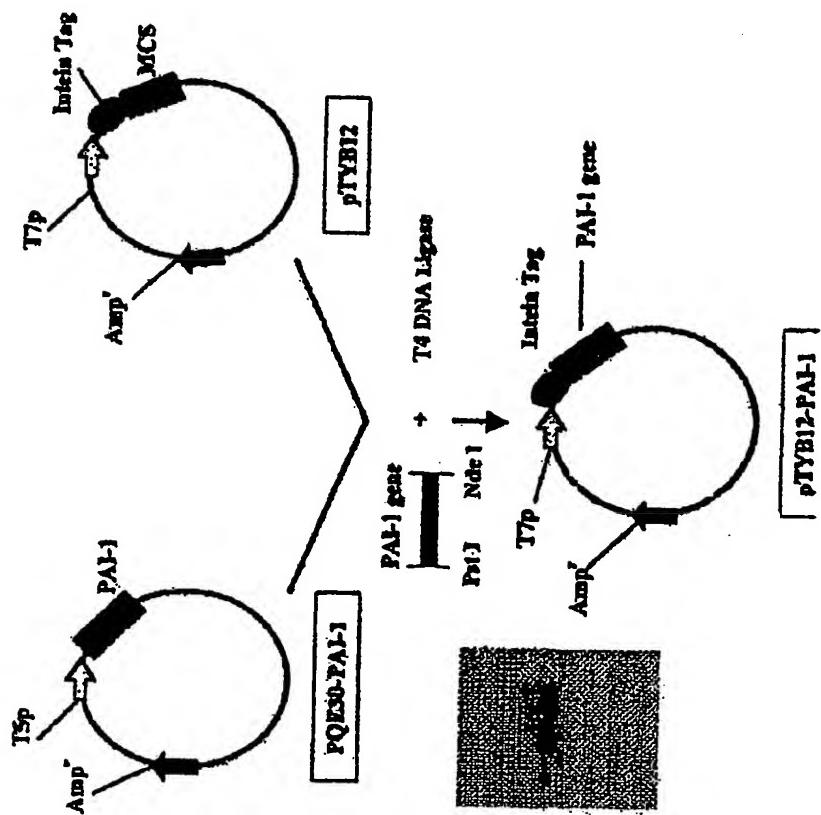


FIG. 7

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FIG. 8B

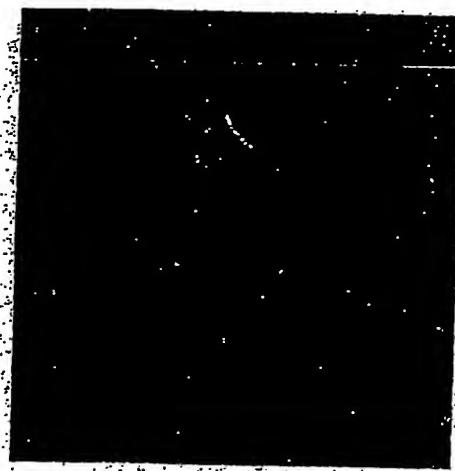


FIG. 8A

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FIG. 9B



FIG. 9A

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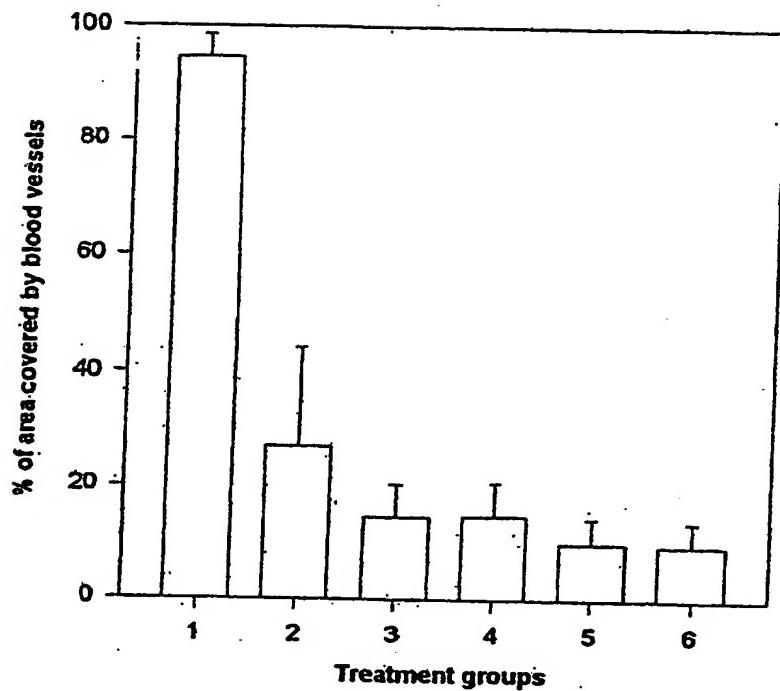


FIG. 10

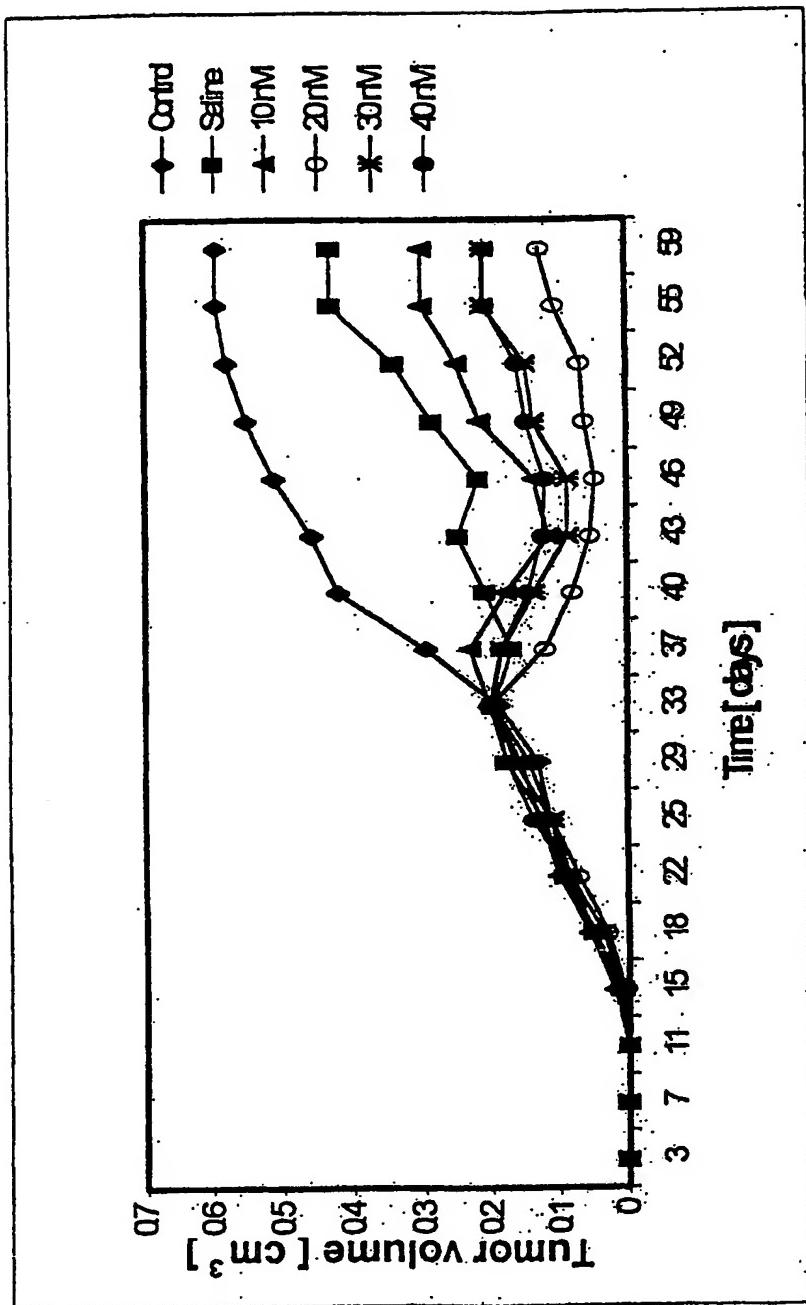


FIG. 11

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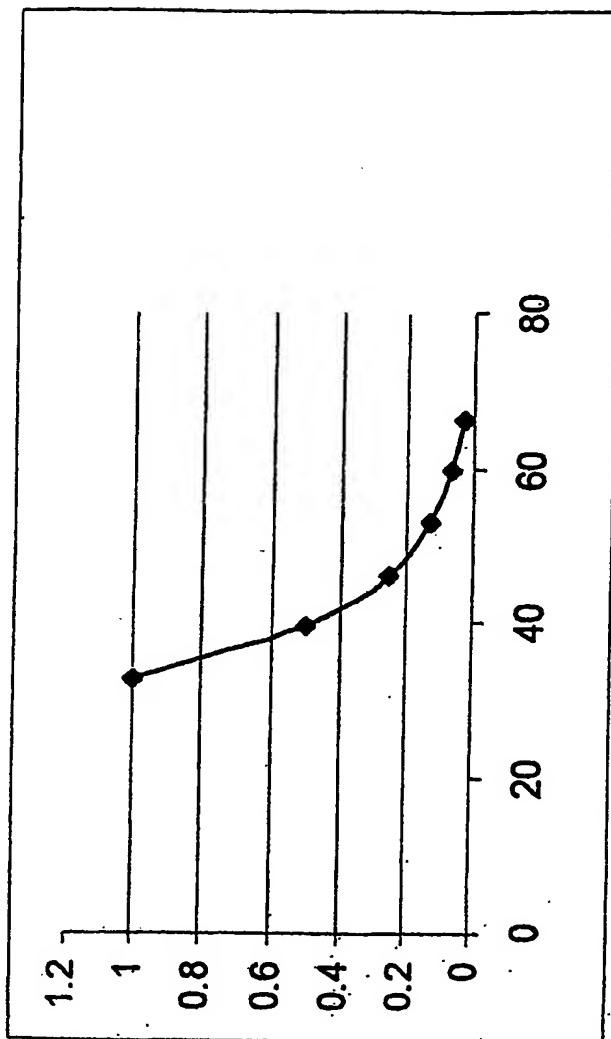


FIG. 12

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